A Study of O-Methylation of Flavonoid Compounds

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ABSTRACT

A Study of O-Methylation of Flavonoid Compounds

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Partially purified enzyme preparations from citrus and tobacco cell culture were used to study the O-methylation pattern of various flavonoid compounds, using S-adenosyl-L-methionine as methyl donor. Enzymatic O-methylation of a number of synthetic and naturally occuring flavonoids was observed at almost all positions of the various hydroxylated flavonoids tested. Enzymatic O-methylation of ring A was readily catalyzed at positions 6, 7 and 8. The degree of methylation was proportional to the electron densities calculated for these positions. Quercetin, a pentahydroxyflavone, was stepwise methylated to a number of methyl products, including several monomethyl (3-,7- and 3'-), dimethyl (3,7-, 7,4'-) and trimethyl (3,7,4'-) derivatives. The results suggested the presence of several O-methyltransferases in these tissues. Results obtained with scutellarein (5,6,7,4'-tetrahydroxyflavone), quercetagetin (3,5,6,7,3',4'-hexahydroxyflavone) and gossypetin (3,5,7,8,3',4'-hexahydroxyflavone) indicate that the biogenesis of the polymethylated flavones of citrus is unlikely the result of methylation of their respective hydroxy analogs, but rather suggest an alternating process of hydroxylation and methylation of partially methylated intermediates. Based on the pattern of O-methylations observed a scheme is proposed for the biosynthesis of partially methylated flavonoids.

A callus tissue culture was initiated from mature orange flavedo to study its potential for flavonoid synthesis. The results demonstrated the initiation and continued growth of callus tissue from orange flavedo. Illumination of the callus tissue stimulated both cell division and tissue differentiation, indicated by lignification and flavonoid production. Callus tissue grown in the dark lacked these features. These differences appear to be linked to the high activity of phenylalanine-ammonia lyase (PAL) in the light-treated tissue. The O-methyltransferase (OMT) activity parallels that of PAL.

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A. INTRODUCTION

Carbon dioxide enters the metabolic carbon pool as a result of the primary synthetic process, photosynthesis. Further metabolic alterations of the photosynthetic products (Fig. 1) lead to the formation of a pool of organic compounds which are precursors of other complex molecules known as the "secondary plant metabolites". Notable among these are the flavonoid compounds which comprise pigments, anthocyanins; the yellow pigments, flavones and flavonols, and the anthochlor pigments, chalcones and aurones (Fig. 2). Flavones and flavonols are interesting in view of their wide distribution and structural variation.

Two important reactions involved in the biosynthesis of flavonoid compounds are hydroxylation and O-methylation. A structural feature of most flavonoids is the common presence of hydroxyl groups at C5 and C7 of ring A, and C4' of ring B; less often at C5, C2', C6 and C8 (Fig. 2c). The biogenetic origin of the C5, C7, C3' and C4' hydroxyl groups can be traced to the acetate/malonate (Birch, 1965) and shikimate (Davis, 1955) pathways of biosynthesis of both aromatic rings, respectively. Modification of the oxygenation pattern of ring-A may proceed through additional hydroxylation at C6, C8, or at both positions.

Almost half of the naturally occurring flavones and flavonols are O-methylated at the hydroxyls of rings A and B, with the ratio of methylation of rings A/B being about one, although the methyl substitution frequencies of ring-A hydroxyls of both groups of

compounds are slightly different. The order of methylation frequency for flavones appears to be C8 >> C6 > C7 > C5 (Venkataraman, 1975), whereas that of flavonols is C6 >> C8 > C7 > C5 (Gottlieb, 1975).

Enzymatic O-methylation and its specificity for ring B of flavonoid compounds has been reported by a number of workers (Finkle and Masri, 1964; Ebel et al., 1972; Wengenmayer et al., 1974; Poulton et al., 1976; Sutfeld and Wiermann, 1978; Tsang and Ibrahim, 1979). On the other hand, very little information is available on ring A methylation.

The common occurrence of partially and fully methylated flavonoids in plants (Wollenweber and Dietz, 1983), as well as the lack of knowledge of ring A O-methylation, prompted us to investigate the O-methyltransferase system in citrus tissues. The latter is known to accumulate a number of tetra to hepta methoxyflavones (Kefford and Chandler, 1970; Swift, 1967).

The aim of the present work was to establish a callus tissue/cell suspension culture of citrus peel and study its potential for the biosynthesis of polymethylated flavones. It was also considered of interest to demonstrate the enzymic O-methylation of rings A and B of a variety of flavonoid compounds using enzyme preparations of intact and in vitro grown tissues. The availability of a number of synthetic flavonoid compounds made it possible to study the sequence of methylation at different positions of the flavonoid ring system in comparison with the methylation known in naturally occurring compounds.

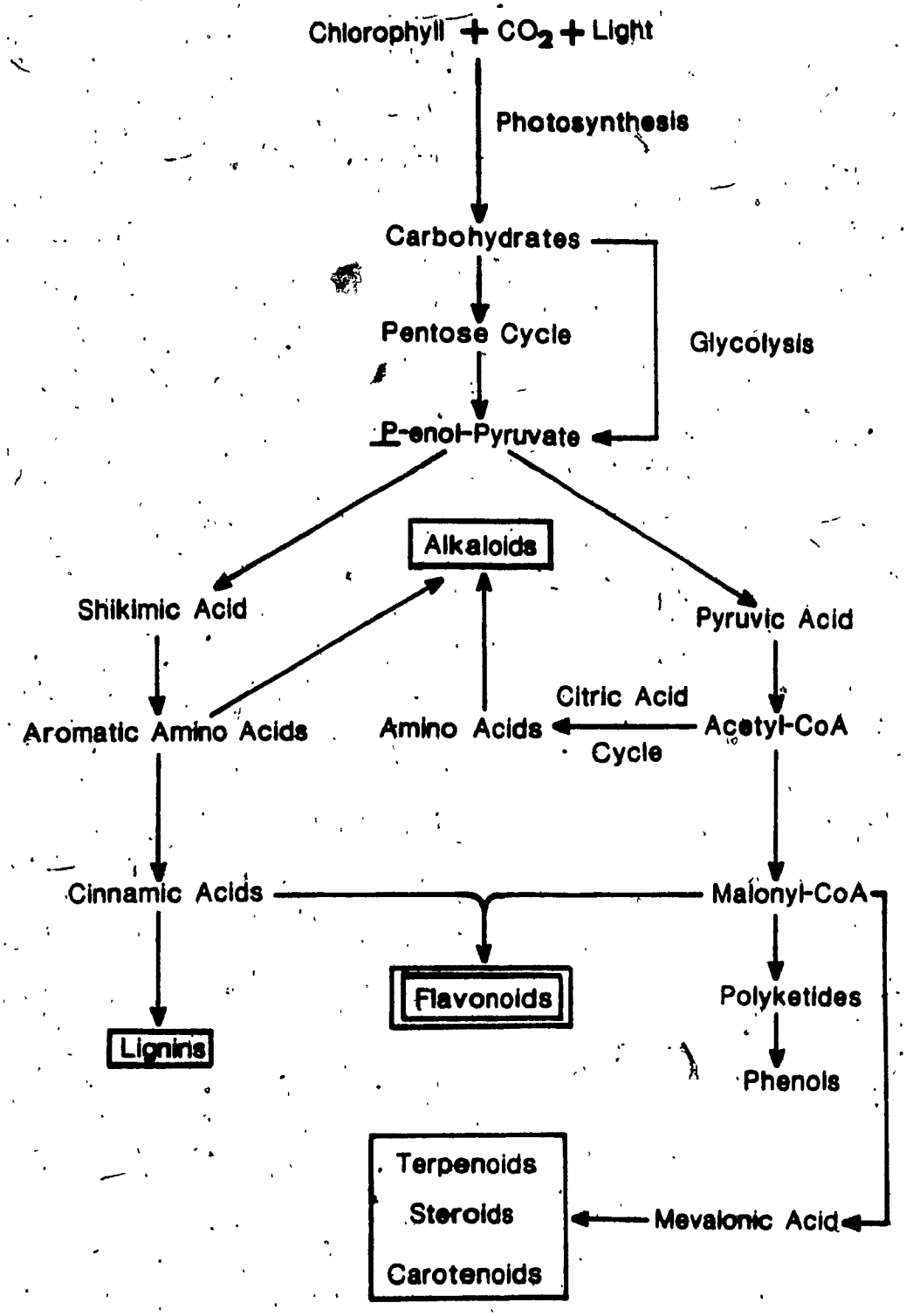


Fig.1 General Pathway For The Formation Of Secondary Plant Metabolites

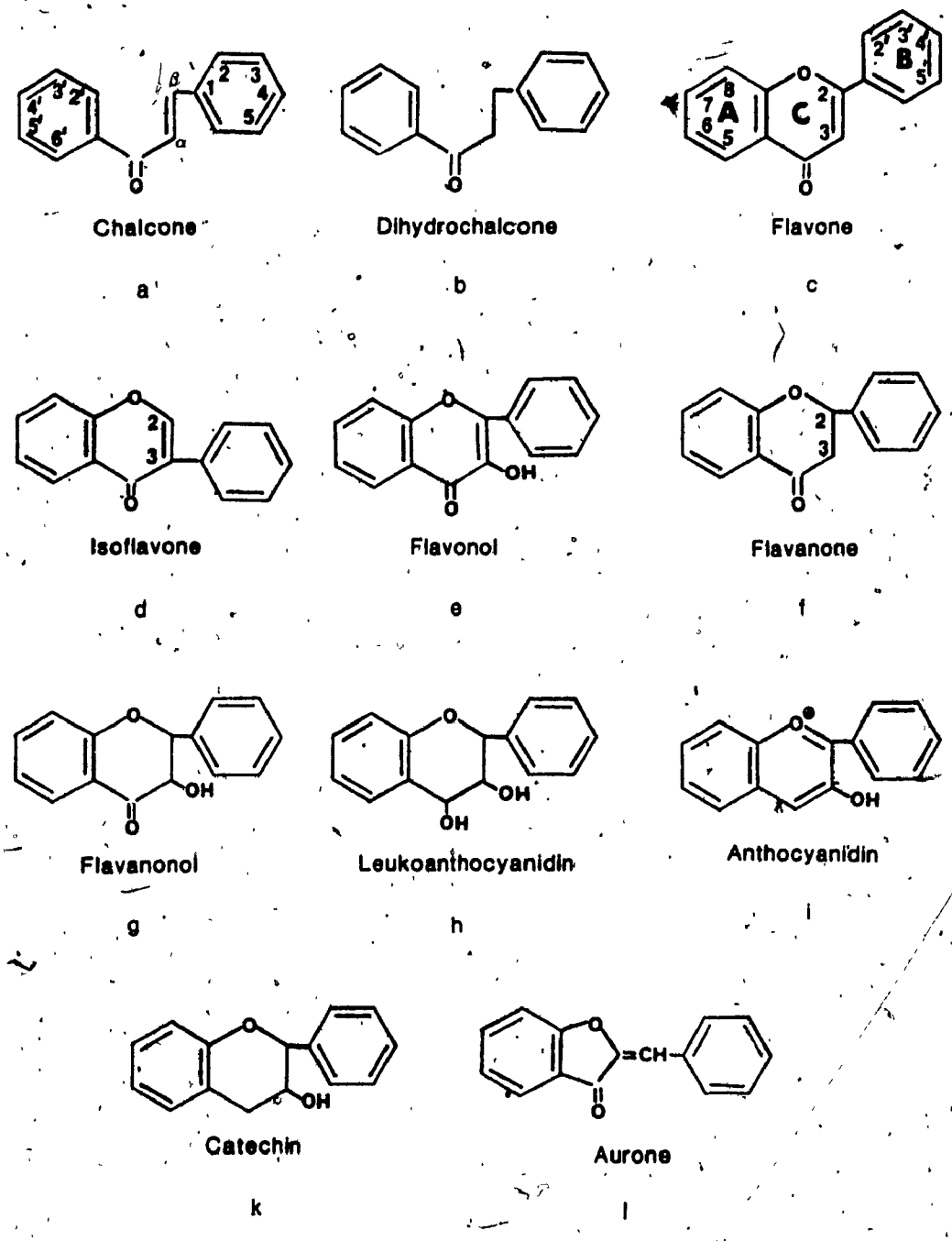


Fig.2 Structures of the Different Groups of Flavonoids

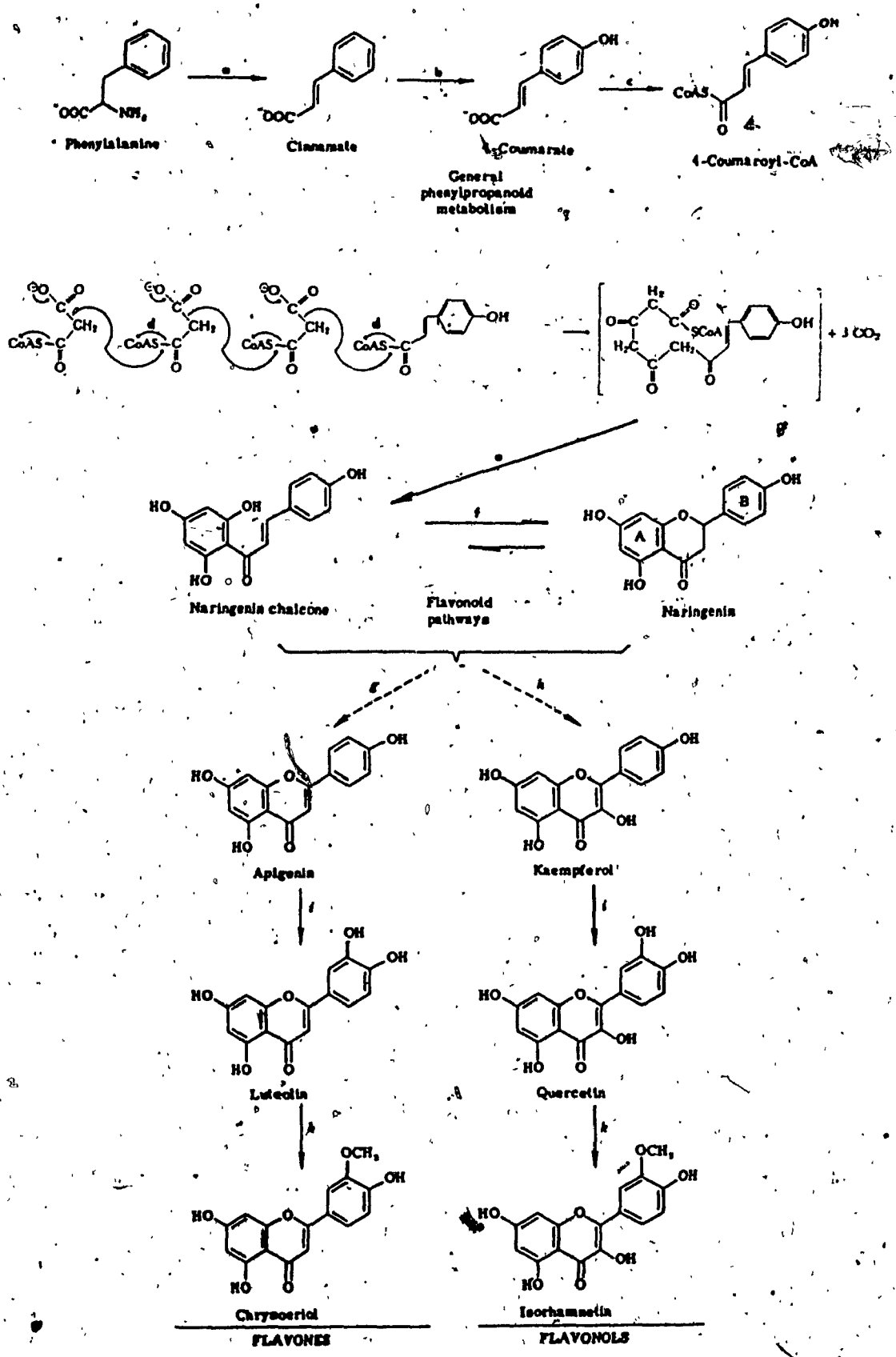
B. REVIEW OF LITERATURE

B.1 Biosynthesis of Flavonoids

Early isotopic experiments (reviews by Neish, 1964, Grisebach, 1965, 1967, 1968) indicated that flavonoids originate from three acetate units by head-to-tail condensation resulting in ring A (Birch and Donovan, 1953), and a phenylpropanoid intermediate derived from the shikimate pathway (Davis, 1955) forms ring B and carbon atoms 2, 3 and 4 (Fig. 3). There is controversy as to whether the substitution pattern of ring B is determined prior to, or after the C15 flavonoid formation. The cinnamic acid starter hypothesis (Hess, 1968) favors the view that a specific ring B substitution is determined prior to the formation of the flavonoid skeleton; caffeic acid (3,4-dihydroxy-cinnamic acid) would be the precursor of 3',4'-dihydroxy-flavonoids. Whereas results of feeding experiments were found to support this view (Hess, 1964; Steiner, 1970; Ebel et al., 1970), much evidence has been accumulating from tracer experiments (Grisebach and Grambow, 1968) and enzymic studies (Grisebach and Hahlbrock, 1974) indicating that modification in the substitution pattern of ring B may occur at a later stage of flavonoid biosynthesis.

B.2 Enzymology of Flavonoids

The enzymes involved in the biosynthesis of hydroxycinnamoyl CoA, a flavonoid ring B precursor, belong to the group associated with general phenylpropanoid metabolism (Group I). These enzymes (Fig. 3) namely PAL (phenyalanine-ammonialyase)(E.C. 4.3.1.5); cinnamate-4-



Adapted from Grisebach (1967) and Hahlbrock (1981)

Fig. 3 Pathway of Flavonoid Biosynthesis

Fig. 3 Continued

Group	Reaction	Enzyme	E.C. Number
I	a	Phenylalanine ammonia-lyase	4.3.1.5
I	b	Cinnamate 4-hydroxylase	1.14.13.11
I	c	4-Coumarate:CoA ligase	6.2.1.12
II	d	Acetyl-CoA carboxylase	6.4.1.2
II	e	Chalcone synthase	-
II	f	Chalcone isomerase	5.5.1.6
II	g	"Flavonoid oxidase 1"[e]	-
II	h	"Flavonoid oxidase 2"[d]	-
II	i	"Flavonoid 3'-hydroxylase"[d]	-
II	k	S-Adenosylmethionine:flavonoid 3'-O-methyltransferase	2.1.1.42

hydroxylase and 4-hydroxy cinnamate CoA ligase, have been recently reviewed (Hahlbrock and Grisebach, 1975; Grisebach and Hahlbrock, 1974). The enzymatic synthesis of flavonoids was demonstrated in parsley cell suspension cultures to proceed *via* chalcone synthase, chalcone-flavanone isomerase (E.C. 5.5.1.6), flavanone and dihydroflavonol oxidase (Stötz and Forkman, 1982); a soluble hydroxylase has been shown to catalyze the 3-hydroxylation of flavonones to dihydroflavonols in *Mathiola* flowers (Forkman *et al.*, 1980). *O*-Methylation and *O*-glycosylation of the resulting flavones or flavonols have been shown to be later steps in flavonoid biosynthesis (Fig 3). These enzymes (Group II) have recently been reviewed by Hahlbrock (1981), Hahlbrock *et al.* (1983), Hösel (1981) and Poulton (1981).

B.3 Flavonoid *O*-Methyltransferases

Enzymatic *O*-methylation involves the transfer of methyl groups from S-adenosyl-L-methionine to the hydroxyl groups of flavonoid compounds. Recent work indicated the presence of two *O*-methyltransferases: one specific for caffeic acid and the other specific for flavonoids. These enzymes have been reported from parsley (Ebel *et al.*, 1972) and soybean (Poulton *et al.*, 1977) cell cultures, as well as tulip anthers (Sütfeld and Wiermann, 1978).

The enzymic methylation of flavonoid compounds has been shown to involve the 3'-position of flavones (Ebel *et al.*, 1972) and flavonols (Poulton *et al.*, 1977; Sütfeld and Wiermann, 1978; Tsang, 1978) and the 4'-position of isoflavones (Wengenmayer *et al.*, 1974),

all concerning ring B. On the other hand, O-methylation of ring A has not been reported until very recently. Poulton et al. (1977) reported the efficient methylation of texasin (6,7-dihydroxy-4'-methoxy-isoflavone) with a purified soybean O-methyltransferase, though the exact position of methylation was not determined. Tsang (1978) demonstrated the 7-O-methylation of quercetin to rhamnetin with a highly purified O-methyltransferase from tobacco cell culture. More recently, a 7-O-methyltransferase has been shown to catalyse one of the steps of a methylation sequence in Chrysosplenium americanum (De Luca and Ibrahim, 1982); this enzyme accepted 3-methylquercetin for further O-methylation to its 3,7-dimethyl derivative. Furthermore, a flavonol 8-O-methyltransferase has been shown to catalyze a terminal step of methylation in Lotus flowers (Jay et al., 1983).

B.4 Biological Roles of Flavonoids

Whereas secondary plant products have been considered for a long time as metabolically inactive waste (Reznik, 1960; Mothes, 1969), the use of more refined techniques has furnished evidence for their dynamic metabolic state (Hösel et al., [illegible]). An extensive review by McClure (1975) has dealt with the biol[illegible]al roles of flavonoids. Some of these functions are outlined in the following sections.

B.4.1 Effect of light

Hahlbrock et al. (1971) have pointed out that light controls flavonoid biosynthesis, and demonstrated its effect on the enzymes leading to apiin synthesis in parsley cell suspension cultures. It

was also suggested that flavonoids may act as a light screen (Nagornaya and Kotsur, 1970; Zenk, 1968) to protect the plant against ultraviolet radiation (Caldwell, 1971). The high flavonoid concentration of tropical plants and those of alpine regions tend to support this contention.

B.4.2 Inhibition of enzymes

Flavonoids are well known to be potent inhibitors of certain enzymes and have been shown to inhibit protein synthesis in vitro (Parups, 1967), as well as inhibition of ribonuclease activity (Van Sumere et al., 1972). They were reported to inhibit the NAD-linked electron transport in oxidative phosphorylation (Ernster et al., 1963; Low and Vallin, 1963) and catechol-O-methyltransferase (Schwabe and Flohe, 1972).

B.4.3 Growth regulation

Another important area not fully elucidated is the function of flavonoids as growth regulators (for a review see McClure, 1975). Rutin was found to abolish the inhibitory effect on plants by some growth substances, while apigenin had little effect (Mashtakov et al., 1971). Naringenin caused bud dormancy and its concentration diminished prior to bud opening (Hendershott and Walker, 1959). Phloridzin and its aglycone, phloretin, inhibited growth and seed germination (Steward and Krikorian, 1971; Podstolski and Lewak, 1970). Naringenin and hesperidin prolong dormancy in citrus seedlings (Feldman et al., 1966). Stenlid (1963) showed that different substitution of hydroxyl groups of flavonoids either inhibited or promoted IAA oxidase activity.

B.4.4 Pathogenicity

Certain plants respond to pathogenic attacks by synthesizing substances known as phytoalexins (Cruickshank and Perrin, 1965). Pisatin, phaseolin and glyceollin are known isoflavonoids produced by the pod endocarp tissues of peas, beans and soybeans respectively, in response to attack by fungi (Cruickshank and Perrin, 1961; 1963). Such compounds are believed to be of pathological significance as they inhibit fungal development in hypersensitive tissue (Müller, 1966) and inhibit germination of fungal spores. Nobiletin and tangeretin show a fungistatic effect in "mal secco" disease of citrus trees. These substances have been found in peel, leaf and bark tissues of resistant citrus varieties (Ben Aziz et al., 1962; Ben Aziz, 1967). Other polymethylated favones associated with resistance (5,4'-dihydroxy-6,7,8,3'-tetramethoxy-flavone and 5,4'-dihydroxy-6,7,8-trimethoxy-flavone) have also been found in citrus trees (Pinkas et al., 1968).

B.4.5 Turnover and degradation of flavonoids

Zaprometov (1977) reviewed the catabolic degradation of flavonoids and the results indicated that whereas ring B is resistant to degradation, ring A is usually degraded to aliphatic compounds and eventually to CO_2 (Patschke et al., 1964; Barz et al., 1974).

B.5 Pharmacological Significance of Flavonoids

Flavonoids came into prominence as "vitamin P" (Ruszniak and Szent-Györgi, 1936), though later studies discredited this role (Szent-Györgi, 1938; Sinclair, 1961). Several reviews by Böhm (1959)

De Eds (1968) and McClure (1975) have described a wide range of effects of flavonoids, some of which were quite controversial. Recently, however, flavonoids again received attention (Foreman, 1984).

Disodium cromoglycate (Intal), a substance structurally related to flavonoids has proven its efficacy in the management of allergic asthma (Brogden et al., 1974). A number of xanthones have been tested on the inhibition of intestinal anaphylaxis and IgE mediated passive cutaneous anaphylaxis. Some of the compounds compared well in their inhibition potency with standard drugs, and have been suggested to be useful in human food allergies (Byars and Ferraresi, 1980). Fewtrell and Gomperts, (1977) have shown that some flavonoids affect the efficiency of the membrane transport ATPases thus interfering with Ca flux, and that quercetin inhibits the antigen-induced release of histamine from mast cells (Fewtrell and Gomperts, 1977a). Bennett et al. (1981) tested 30 natural flavonoids and observed that the inhibitory effect on rat mast cell secretion involved receptors directed at the calcium channels, whereas for rabbit neutrophils no significant changes in calcium movement across the plasma membrane were noted. Middleton et al. (1981) showed that quercetin inhibited the ragweed stimulated histamine release from human basophils but did not affect cyclic AMP levels. The different effects observed of quercetin on calcium flux and clyclic AMP, could be due to concentration effects. Ennis et al. (1980) have proposed that quercetin at low concentrations prevents Ca^{++} influx, while at higher concentrations, acts by elevating intracellular cyclic-AMP levels. Flavonoids may not have a common

mechanism of action, but by binding to IgE, IgG, calcium channel receptors, or elevation of cyclic AMP as well as phosphodiesterase inhibition, would all result in inhibition of the release mechanism.

C. MATERIALS AND METHODS

C.1 Plant Material

The following Citrus species were used in this investigation:

1. Orange: Citrus sinensis var. Washington Navel and Valencia.
2. Grapefruit: Citrus paradise var. White River.
3. Lemon: Citrus limon.
4. Orange hybrid: Citrus mitis var. Calamondin.

The fruits of 1-3 were obtained from commercial sources. Mature plants of Calamondin were bought from local nurseries and placed in the Department's greenhouse. These were used as the source of fresh fruits for enzyme preparations. Callus tissue cultures were initiated from fruit and seed.

Seeds from C. sinensis and C. mitis were germinated and the seedlings used as source of O-methyltransferase.

Nicotiana tabacum cell suspension was used from the stock culture, maintained in this laboratory.

C.2 Initiation of Citrus Peel Callus Cultures

Citrus fruits were washed with detergent, rinsed and surface-sterilized by immersion for 15-20 minutes in a diluted solution of Chlorox or Javex (5-12% available Cl_2). The fruit was then rinsed with sterile water and was finally dipped for several minutes into 70% ethanol. Further manipulations were carried out under aseptic conditions in a laminar flow hood. Sections of the fruit peel were removed and cut into strips of approximately 10 x 2 mm, and the albedo

tissue was excluded. Explants consisting of flavedo tissue were placed onto a salt-nutrient culture medium (Murashige and Skoog, 1962) containing the following addenda (mg/L) (Murashige and Tucker, 1969; Brunet and Ibrahim, 1973) sucrose, 50,000; myo-inositol, 100; glycine, 2; thiamin-HCl, 10; nicotinic acid, 5; pyridoxin-HCl, 10; indoleacetic acid, 10; kinetin, 1; casein hydrolyzate, 1000; and 20% v/v coconut water. The medium was solidified with 0.7% agar and its pH adjusted to 5.6 - 5.8. Twenty-five ml aliquots were dispensed into 8 oz. prescription bottles and autoclaved for 15 minutes at 15 p.s.i.

The tissue explants were kept in the dark until good callus development had taken place (6-8 weeks). Subcultures were initiated at 6-8 week intervals using the same medium. Callus tissue growth was maintained at 30 ± 1.0°C in a growth chamber in the dark or under continuous illumination of about 3000 lux.

C.3 Extraction of Flavonoids from Fruit Peel and Callus Tissue

Citrus tissues, fruit peel and light or dark-grown callus were dried in a vacuum oven at 40°C, then ground to a fine powder. Known weight of either powder was exhaustively extracted with benzene in a Soxhlet extractor. The benzene extract was washed several times with 1% NaOH to remove acidic substances, and subsequently evaporated. The residue was dissolved in water and the volatiles removed by flash-evaporation. The residue from the latter treatment was taken up in ethanol - 5% NaOH (1:1) and allowed to stand for two hours in order to convert the lactones into their acid salts. The mixture was then further diluted with water and extracted with benzene to separate the

flavonoids. The benzene extract was evaporated to dryness and used for chromatography.

C.3.1 Chromatography of flavonoid extracts

Two-dimensional separation was carried out on commercially prepared silica gel-G plates (E. Merck, Darmstadt). The solvent systems consisted of hexane-n-butanol (85:15 v/v) for the first direction and benzene - acetone (9:1 v/v) for the second (Swift, 1967). The plates were developed twice in each direction.

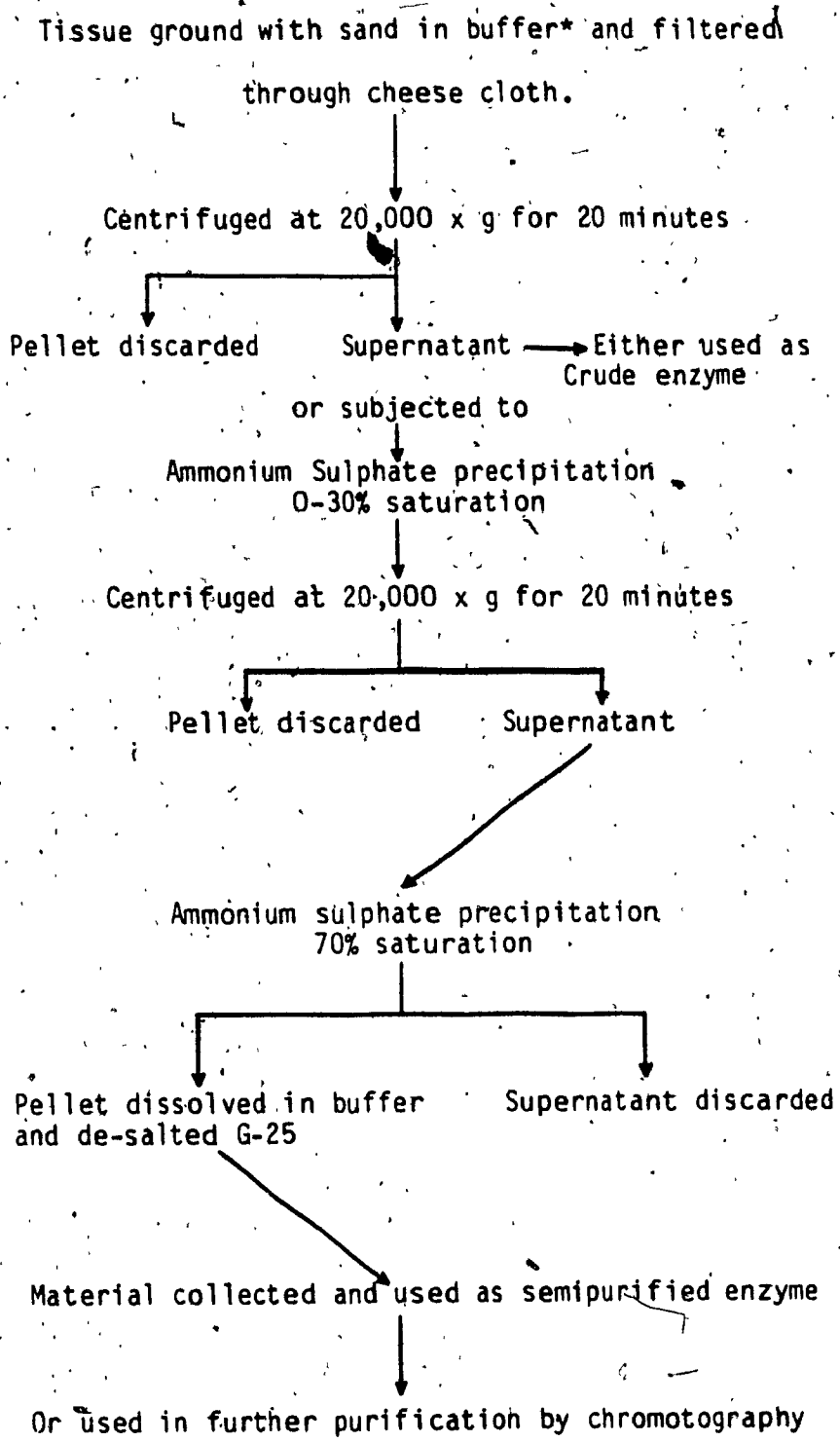
C.3.2 Identification and quantitation of flavonoids

The flavonoid compounds were located by their characteristic fluorescence in U.V.-light (366 nm) and their UV-absorption maxima were determined by use of spectral shift reagents (Mabry et al., 1970). Identification was further confirmed by co-chromatography with authentic samples and comparison of their spectral data. Since the amounts of flavonoids found in calli were extremely low, they were determined by a semi-quantitative method based on comparison of their fluorescence-intensity and of spot size with those of standard compounds chromotographed at different concentrations (ca. 1-25 µg).

C.4 Extraction and Purification of O-Methyltransferase

A summary of the procedures used is shown in Figure 4. The following buffers were used (Dawson et al., 1962):

1. 0.05M phosphate buffer - pH 7.4 (KH_2PO_4-NaOH pH 5.8 - 8.0)
2. 0.05M Tris-HCl buffer - pH 7.4 (pH 7.2 - 9.1)



*If semipurified enzyme was used, the initial buffer during grinding contained 0.05M EDTA.

Fig. 4 Flow Sheet for OMT Extraction and Purification

Extraction and purification of O-methyltransferase were carried out in the cold room at 2-4°C. The plant material was rinsed with water and dried between paper towels. Cell cultures were filtered and cells were thoroughly washed with distilled water. The tissues were ground in a chilled mortar using fine sand, Polyclar AT(0.1% w/v) (Serva, Heidelberg) and ice cold buffer (1:3 w/v). The homogenate was filtered through Nitex (25 μ) (Tober, Ernst and Traber Inc., Elmsford, U.S.A.) and the filtrate was centrifuged for 20 minutes at 20 000 x g. The supernatant was then stirred for 20 minutes with Dowex 1 x 2 (Sigma, U.S.A.), which had been equilibrated with the same buffer.

C.4.1 Ammonium sulphate fractionation

When a partially purified enzyme preparation was used, the crude extract was subjected to ammonium sulphate precipitation. The protein which precipitated between 30-70% saturation was collected by centrifugation.

C.4.2 Column chromatography

Sephadex G-25 (Pharmacia, Uppsala, Sweden) beads were allowed to swell for 5 hours, or overnight in the cold with 0.05M Tris buffer, pH 7.4. The fines were discarded and the gel was loaded into a 2 x 25 cm column. The column was further washed with several volumes of buffer for equilibration.

The ammonium sulphate pellet was dissolved in the minimum amount of buffer, applied to the column and the protein was eluted with the same buffer using a UV-monitor (Model 100, Pharmacia).

C.5 O-Methyltransferase (OMT), Assay and Identification of Reaction Products

C.5.1 Standard enzyme assay

OMT activity was measured by an isotopic assay, using [^{14}C-methyl]-S-adenosyl-L-methionine (SAM), (57.8 mCi/mmole New England Nuclear) as the methyl donor. The standard reaction mixture consisted of 30-60 nmole substrate dissolved in 10-20 µL dimethylsulphoxide (DMSO), 10 nmole SAM containing about 55'000 dpm, 100-200 µL enzyme protein in Tris-HCl buffer, pH 7.4. Control assays contained no substrate, but 10-20 µL DMSO. The assay mixture was incubated in 1.5 mL Eppendorf microtubes at 35°C for 30 minutes and the reaction was stopped by addition of 20 µL 6N HCl.

The reaction mixture was extracted with 0.7 mL of benzene-ethylacetate (1:1) by shaking on an Eppendorf rotary shaker (Model 3200) for 10-15 minutes. The separation of the organic phase was achieved by centrifugation at full speed for 2 minutes in an Eppendorf microcentrifuge (Model 3300). The organic layer was collected and 200 µL aliquots from each tube were transferred to liquid scintillation vials, to which 0.3 mL absolute ethanol and 10 mL liquid scintillator were added. The mixture was then shaken and counted for radioactivity. The rest of the assay products were evaporated, redissolved in 100 mL MeOH and used for chromatography. Experiments were usually conducted in duplicates.

C.5.2 Identification of reaction products

The reaction products were evaporated and redissolved in a mixture of dry ethanol, benzene and ethylacetate (1:2:2). The dissolved reaction products were applied to TLC-plates, along with authentic substances whenever available (Table I), and chromatographed with the appropriate solvent systems either one or two-dimensionally. The different chromatographic systems and their composition are listed in Table II. After chromatography, the plates were dried in a fume hood then placed in contact with Kodak No-Screen X-ray film (8 x 10 inches, Eastman Kodak Co., Rochester, USA). Exposure time varied from 5 days to several weeks, depending on the amount of radioactivity. Following exposure, the films were developed and the radioactive spots were recorded by contact printing.

Identification of the products was based on co-chromatography with authentic samples, or comparison of their Rf values in different solvent systems with those published (Jay et al., 1975; Bohm et al., 1977).

C.5.3 Liquid scintillation counting

Radioactive spots were carefully scraped off the TLC-plates and transferred to glass scintillation vials containing 10 mL liquid scintillator (5 g PPO/L of toluene, New England Nuclear) and sufficient quantities of Cab-O-Sil (Cabot Corporation, USA) to form a gel. Samples were counted either in a Beckman (Model LS-255) or Unilux II - (Nuclear Chicago, Illinois) beta-counter.

Table I - Source of Flavonoid Substrates and Products of OMT Used in this Investigation.

	Flavone/isoflavone and Substitution	OH	OME	Source
1.	3-Hydroxyflavone	3	-	Pfaltz & Bauer
2.	3-Methoxyflavone	-	3	Synthesis
3.	7-Hydroxyflavone	7	-	ICN Pharmaceuticals
4.	5,6-Dihydroxyflavone	5,6	-	Merck-Frosst
5.	5-Hydroxy-6-methoxy-flavone	5	5,6	Synthesis
6.	5,7-Dihydroxyflavone	5,7	-	Roth, Pfaltz & Bauer
7.	7,8-Dihydroxyflavone	7,8	-	Synthesis
8.	7-Hydroxy-8-methoxy-flavone	7	8	Synthesis
9.	7,8-Dimethoxyflavone	-	7,8	Synthesis
10.	Galangin	3,5,7	-	Roth
11.	3-Methylgalangin	5,7	3	Gift (G. Hrazdina)
12.	Baicalein	5,6,7	-	Gift (J. Poulton)
13.	Norwogonin	5,7,8	-	Synthesis
14.	5,7,8,4'-Tetra-OH-flavone	5,7,8,4'	-	Synthesis
15.	5,7,8-Tri-OH-4'-OCH_3-flavone	5,7,8	4'	Synthesis
16.	6,7,4-Trihydroxy-isoflavone	6,7,4'	-	Pfaltz & Bauer
17.	Texasin (iso-)	6,7	4'	Gift (W. Hösel)
18.	8-Hydroxyacacetin	5,7,8	4'	Synthesis
19.	Apigenin	5,7,4'	-	Roth; Aldrich
20.	Acacetin	5,7	4'	Aldrich
21.	Scutellarein	5,6,7,4'	-	Gift (M. Jay)
22.	Tetra-O-methyl-flavone	-	5,6,7,4'	Gift (L.J. Swift)

23.	Luteolin	5,7,3',4'	-	Roth
24.	Chrysoeriol	5,7,4'	3'	Roth
25.	Diosmetin	5,7,3'	4'	Merck
26.	Kaempferol	3,5,7,4'	-	Roth; Fluka
27.	Kaempferid	3,5,7	4'	Roth
28.	Dihydroquercitin	3,5,7,3',4'	-	Merck
29.	Quercetin	3,5,7,3',4'	-	K & K; Baker
20.	3-Me-quercetin	5,7,3',4'	3	Gift (L. Jurd)
31.	Rhamnetin	3,5,3',4'	7	Roth; Synthesis
32.	Isorhamnetin	3,5,7,4'	3'	Roth; Synthesis
33.	Tamarixetin	3,4,7,3'	4'	Synthesis
34.	3,7-Di-Me-quercetin	5,3',4'	3,7	Gift (M. Jay)
35.	Ombuin	3,5,3	7,4'	Synthesis
36.	3,4'-Di-Me-quercetin	3,5,7	3',4'	Pfaltz & Bauer
37.	Ayanin	5,3'	3,7,4'	Gift (M. Jay)
38.	Retusine	5	3,7,3',4'	Gift (M.Jay)
39.	Quercetagetin	3,5,6,7,3'4'	-	Roth
40.	3,7-Di-Me-quercetagetin	5,6,3',4',3,7		Gift (M. Jay)
41.	3,6,7-Tri-Me-quercetagetin	5,3'4'	3,6,7	Gift (M. Jay)
42.	Gossypetin	3,5,7,8,3'4'	-	Gift (M. Jay)
43.	Tetramethyl-scutellarein	-	5,6,7,4'	Gift (L.J. Swift, R.E.Berry,J.Tatum)
44.	Heptamethoxyflavone	-	3,5,6,7, 8,3',4'	Gift (L.J. Swift, R.E.Berry,J.Tatum)
45.	Nobiletin	-	5,6,7,8, 3',4'	Gift (L.J. Swift, R.E.Berry,J.Tatum)
46.	Sinensetin	-	5,6,7, 3',4'	Gift (L.J. Swift, R.E.Berry,J.Tatum)

Table II - Chromatographic Support and Solvent System

TLC-Plates	Solvent v/v	Authors
Silica Gel (Merck Darmstadt)	Hexane:n-Butanol 85 15	Swift (1967)
	Benzene:Acetone 9 1	
	Benzene:Pyridine:Formic acid 36 18 5	Hörhammer et al. (1964)
	86 19 5	Modified
	Methanol:Chloroform 15 1	Mabry et al. (1970)
Cellulose (Macherey & Nagel)	Acetic acid:H_2O:Benzene 6 4 4	Jay et al. (1975)
Polyamide MN-6 (Macherey & Nagel)	Methylethyl-Ketone:Methanol:Benzene:Petrol ether 3 3 60 26	
	Methylethyl-Ketone:Methanol:Toluene 7 7 60	
	Ethylformate:Ethanol:H_2O:Cyclohexane 20 19 1 25	Bohm et al. (1977)
	Ethylformate:n-Butylacetate:Formic acid 50 23 2	

C.5.4 Protein determination

The protein content of the different fractions of preparation was estimated by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

C.6 Source of Flavonoid Compounds

A large number of flavonoid compounds were used as methyl acceptors with the OMT assay. The majority of compounds were available from commercial sources; some were synthesized in the laboratory by the standard methods, others were generous gifts from a number of colleagues in different laboratories. A list of the names and sources of the flavonoids used in this investigation is given in Table I.

C.6.1 Synthesis of specific flavonoid compounds

7,8-Dihydroxyflavone was prepared by the method of Cramer et al., (1956) and consisted of (a) condensation of galloacetophenone and benzoyl chloride in dry pyridine with the formation of galloacetophenone-tribenzoate, (b) the latter was treated with sodium ethylate in dry benzene and (c) the product was cyclized with acetic acid and sodium acetate. 7,8-Dihydroxyflavone was obtained as pale yellow crystals (m.p. 240 uncorr.) after two recrystallizations and its purity was tested by TLC.

C.6.1.2 5,7,8-Trihydroxyflavone (Norwogonin) was prepared by the oxidation of chrysin (5,7,-dihydroxyflavone) using potassium

persulphate in aqueous potassium hydroxide (Rao et al, 1947; Sethna, 1951) and the mixture was left to stand at room temperature for 24 hours. After slight acidification, the brown solid which separated was filtered off and the filtrate was washed twice with diethylether. The aqueous layer was then treated with sodium sulphite and concentrated HCl, and was kept for 30 minutes at 90°C when a yellow solid separated out. The latter was crystallized from a mixture of ethylacetate and petroleum ether (m.p. 260° uncorr.).

C.6.1.3 8-Hydroxyacacetin (5,7-dihydroxy-4'-methoxyflavone) was oxidized at the 8-position using potassium persulphate in aqueous potassium hydroxide, as described in Section 6.1.2 (Rao et al., 1949). Crystallization from ethylacetate-petroleum ether yielded yellow plates (m.p. 275° uncorr.).

C.6.2 O-methylation of hydroxyflavones

Partial methylation of a number of hydroxyflavones (Table I) was carried out using dimethyl sulphate with anhydrous potassium carbonate in acetone solution (Simpson and Beton, 1954) or with diazomethane in dry pyridine (Fales et al., 1973). Thereby, 5,6-dihydroxyflavone gave the 6- and 5,6-dimethyl derivatives; 7,8-dihydroxyflavone produced the 8- and 7,8-dimethyl derivatives and rutin (quercetin-3-rhamnoglucoside) gave 7-, 4'- and 7,4'- methyl derivatives. The latter were in the form of 3-glycosides, and their aglycones were released after hydrolysis with diluted HCl. Identity and purity of the methylated products were verified by the standard

chromatographic and spectrophotometric techniques (Mabry et al., 1970; Jay et al., 1975).

C.6.3 Presentation of data on O-methylation of flavonoid compounds by cell-free extracts of citrus and tobacco

The results of O-methylation of various substrates used are presented in Tables VI-IX. Total incorporation of label into the reaction products is expressed as a percentage of that of quercetin (= 100%) when used as substrate. The activity ratios are based on the counts (cpm) in individual compounds that were scraped off the chromatogram after autoradiography. When no reference compounds were available, tentative identification of products was based on the comparison of their chromatographic characteristics with those published in the literature. With the solvent systems used (Table II), it was generally found that a) the methylated products have higher Rf values than those of their hydroxylated substrates, with the possible exception of the 5-OMe-quercetin which runs only slightly ahead of quercetin, and the penta-OMe-quercetin which runs ahead of the mono-methyl products; b) Rf values of reaction products increased with increasing number of their methoxyl groups; c) compounds with OMe-groups at the meta position (6-,8- or 3'-) exhibited higher Rf values than those with methoxyl groups at the para position (7- or 4'-); d) electron density calculations indicating reactivity of groups due to their relative nucleophilicities. In most cases, labelled products were chromatographed two-dimensionally on one support

(polyamide, silica gel or cellulose). In some cases unresolved compounds were scraped and re-chromatographed on a different TLC support for better resolution.

The electron density calculations served as a good criterion in product identification. The conformation of a molecule is a function of its molecular structure and the understanding of its electronic and conformational structure is essential for the elucidation of its precise mode of action. As the methylation of hydroxyl groups can be conceived as a nucleophilic attack of the oxygen atom on an activated methyl group, there is a reasonable possibility to observe a good correlation between the electronic density of the oxygen atom and its reactivity towards the methyl group. In such a nucleophilic reaction, the interaction between the nucleophile and the elctrophile is essentially one involving the highest occupied molecular orbital (HOMO) with the lowest unoccupied molecular orbital of the electrophile. The interaction between these two orbitals is most important when these orbitals are very close in energy. The CNDO/2 method is a suitable method to estimate the charge distribution in molecule and the energy levels of these orbitals of interest. CNDO/2 has been used successfully in the study of electronic structure of neurotransmittors (Brothwick and Steward, 1977) pyrylium compounds (Martensson and Warren, 1970).

Structures were generated using the Merck Molecular Modeling System (MMMS) (Pople and Beveridge, 1970). Normal CNDO/2 parametrization was used (Pople et al., 1965). Standard bond lengths and angles were used for all the flavone structures that were considered (Hayashi et al., 1974).

D. RESULTS

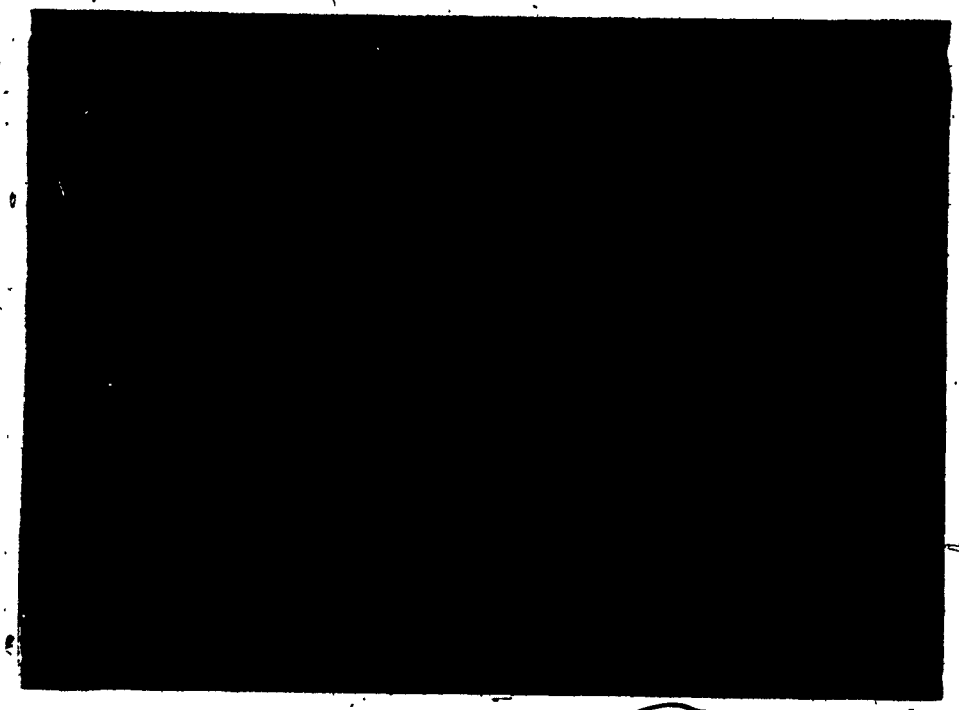
D.1 Growth of *Citrus* Tissue Cultures

The induction of callus tissue on cultured fruit peel explants was determined from several batch cultures. The first signs of callus initiation appeared after 4-5 weeks from culture, and reached maximal growth after another 4-5 weeks (Fig. 5). The rate of success of callus initiation from peel tissue was about 50% for lemon, 25% for grapefruit and 10% for orange. The subcultured callus tissues, either lemon, grapefruit or orange, were maintained on the high vitamin, S-M-medium, and exhibited almost similar growth rates (ratio of final to initial weight) which amounted to a 6-8 fold increase. Coconut water (15% v/v) seemed to be an essential requirement for growth, since poor or arrested growth took place in its absence.

Several attempts were made to initiate a suspension culture from the callus tissue, but were unsuccessful. These attempts included dilution of the culture medium, variation of the amount of growth regulators and the addition of gibberellic acid to the culture medium in order to increase friability of the callus tissue.

D.2 Histology of Callus Tissue

Microscopic examination of sectioned, light-grown callus tissue indicated extensive cell differentiation and the formation of lignified tissue (Fig. 6B) that was responsible for the compact nature of the callus. Similar observations were reported by Dr. Einset, Dept. of Horticulture, University of California (personal communication).



Orange Peel Showing Callus Initiation.

B: 4-5 weeks old. A: 6-7 weeks old. C: 8-10 weeks old (Mag. x 3)

Fig. 5 Orange Peel Calli. Upper row: Grown in the dark

Lower row: Grown in continuous light

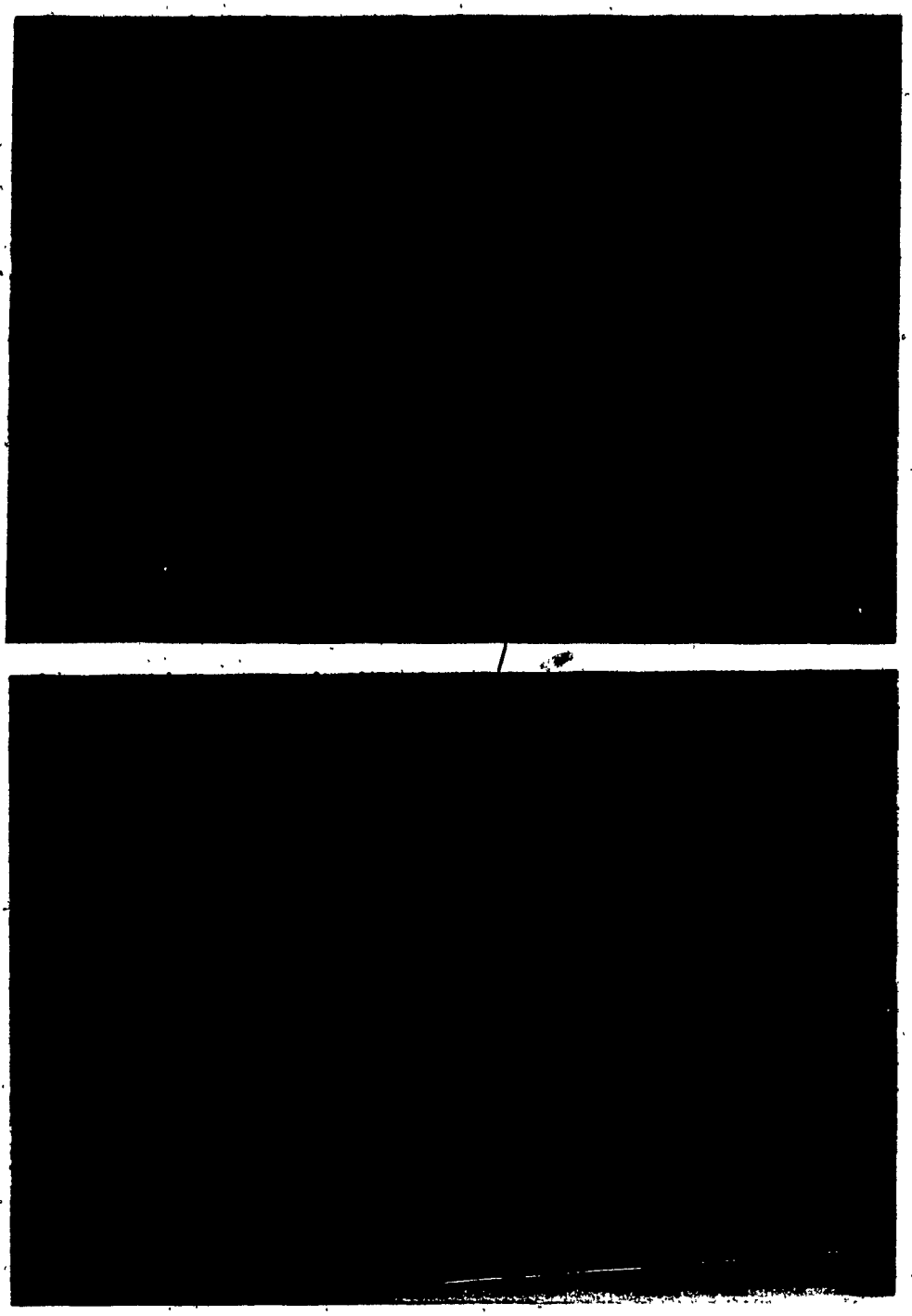


Fig. 6 Sectioned Orange Callus Grown in Continuous Light (ca. 3000 lux)

A: Active cell division (100 x)
B: Cell differentiation and lignification (300 x)

D.3 Flavonoid Composition of Citrus Tissue

D.3.1 Identification of callus tissue flavonoids

Analysis of flavonoid compounds was carried out on 4-5 weeks old callus tissue of the 4th, 5th and 6th subcultures. The flavonoid pattern of light-grown callus tissue consisted of five highly-methoxylated flavones and two unidentified compounds. Their relative position on a two-dimensional chromatogram is shown in Fig. 7; Table III lists their characteristic fluorescence in UV-light and their absorption maxima that were obtained with different spectral shift reagents (Mabry et al., 1970). The lack of spectral shifts with NaOH or $AlCl_3$ (Table III) was indicative of the absence of free hydroxyl groups on the C_{15} ring system, and suggested full methylation of these flavonoids. Addition of a few drops of conc. HCl caused protonation of the heterocyclic ring oxygen, and produced bathochromic shifts of about 40-70 nm (Table III), with tetra-methyl-O-scutellarein, sinensetin and nobiletin. The absence of an acid shift with auranetin and heptamethoxyflavone may have been due to their low concentration in the analysed extracts.

The identity of the orange callus flavonoids was confirmed by co-chromatography with authentic samples (kindly supplied by Drs. Swift, Tatum and Berry, 1967, Florida), except for compound No. 3 which was tentatively identified as auranetin. These compounds are known to occur naturally in orange peel tissue (Horowitz 1961).

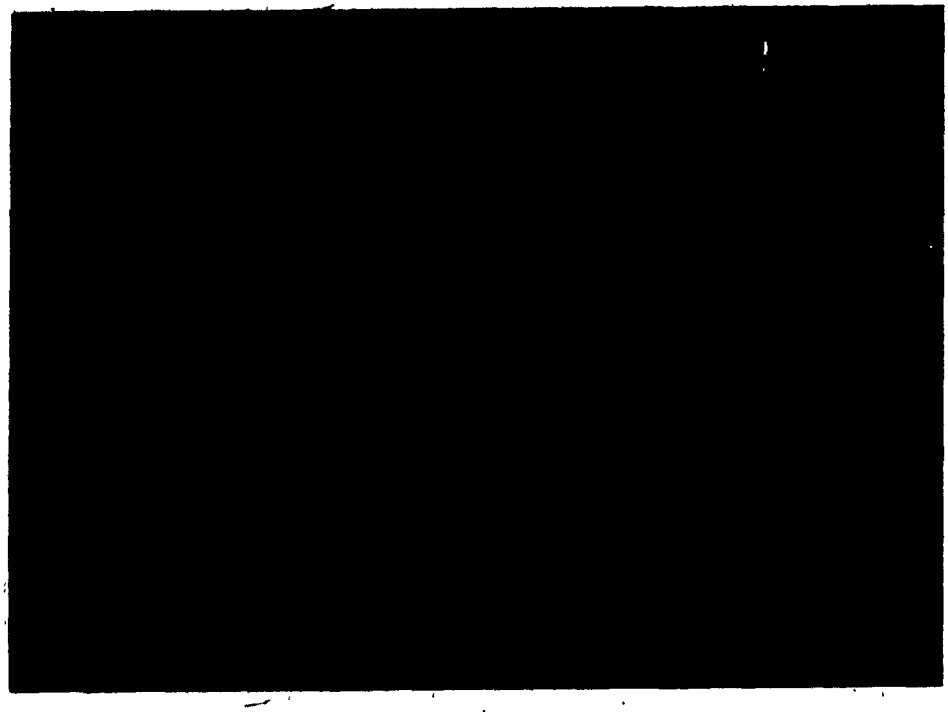


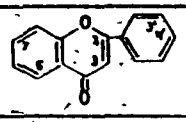
Fig. 7 Two-dimensional Separation of the Neutral Fraction of Orange Flavones on Silica Gel Plates.

First direction: hexane:butanol (85:15)

Second direction: benzene:acetone (9:11)

1, Sinensetin; 2, Auranetin; 3, Nobiletin; 4, Tetramethyl-O-Scutellarein; 5, Heptamethoxyflavone; 6, Tangeretin.

Table III: Chromatographic and Ultraviolet Spectral Characteristics of Flavonoids Isolated from Citrus Callus Tissue



	Rf^1 (x 100)	RF^2	Colour in UV-light (366 nm)	MeOH	MeONa	λmax MeOH/$AlCl_3$	MeOH/conc. HCl
5,6,7,4'-tetramethoxyflavone (Tetramethyl-O-scutellarein)	52	25	Pink	265,320	265,322	265,321	265,328, 394
3,5,6,7,8,3',4'-heptamethoxyflavone	57	39	Green	253,270sh, 341	253,270sh, 341	253,270sh, 342	253,264sh, 341
5,6,7,8,3',4'-hexamethoxyflavone (Nobiletin)	47	24	Greenish-blue	248,268, 333	248,267 333	248,269, 337	250,268, 371
3,6,7,8,4'-pentamethoxyflavone (Auranetin)[3]			Bright blue	242,332	240sh,331	245,342	340
5,6,7,3',4'-pentamethoxyflavone (Sinensetin)	24	18	Grayish-white	240,262sh, 329	240,262sh, 329	242,262sh, 330	239sh,255sh, 335,404
5,6,7,8,4'-pentamethoxyflavone (Tangeretin)	69	37	Yellow	322,270			
A. Unidentified			Blue	not determined			
B. Unidentified			Blue	not determined			

1 Hexane-Butanol (17:3 v/v)

2 Benzene-Acetone (9:1 v/v

3 This is now considered to be a fully methylated quercetagetin (3,5,6,7,3',4'-hexamethoxyflavone) Personal communication by Dr. Tatum, U.S.D.A. Fruit and Vegetable Products Lab. Winter Haven, Florida

sh shoulder or inflection

D.3.2 Comparison of flavonoids of different citrus tissues

A comparison of the flavonoid content of orange peel and its callus tissue (Table IV) indicated their presence in small amounts in callus tissue. However, it is noteworthy that sinensetin and nobiletin constituted the major components of both tissues (Fig. 8). Dark grown tissue, on the other hand, contained only trace amounts of these flavonoids, which could not be quantitated.

The flavonoid pattern of grapefruit peel was qualitatively similar to that of the orange, albeit in lower amounts, especially those of sinensetin and nobiletin. The flavonoid pattern of lemon and its callus, on the other hand, showed no congruence and therefore no attempt was made to identify these flavonoids (Fig. 9).

D.3.3 O-Methyltransferase activity at different stages of calamondin orange fruit development

Immature fruits exhibited low enzyme activity which increased with fruit development reaching a maximum before the onset of yellowing, decreasing steadily thereafter (Table V).

Table IV: Amounts of Flavonoids in Mature Orange Flavedo and its Callus Tissue

Flavonoid compound	µg/g dry tissue	
	Flavedo	Callus
Tetramethyl-O-scutellarein (5,6,7,4'-tetramethoxyflavone)	104	6.2
3,5,6,7,8,3',4'-Heptamethoxyflavone	102	2.4
Nobiletin (5,6,7,8,4'-hexamethoxyflavone)	276	19.2
Auranetin (3,6,7,8,4'-pentamethoxyflavone)	138	5.2
Sinensetin (5,6,7,3',4'-pentamethoxyflavone)	260	32.5

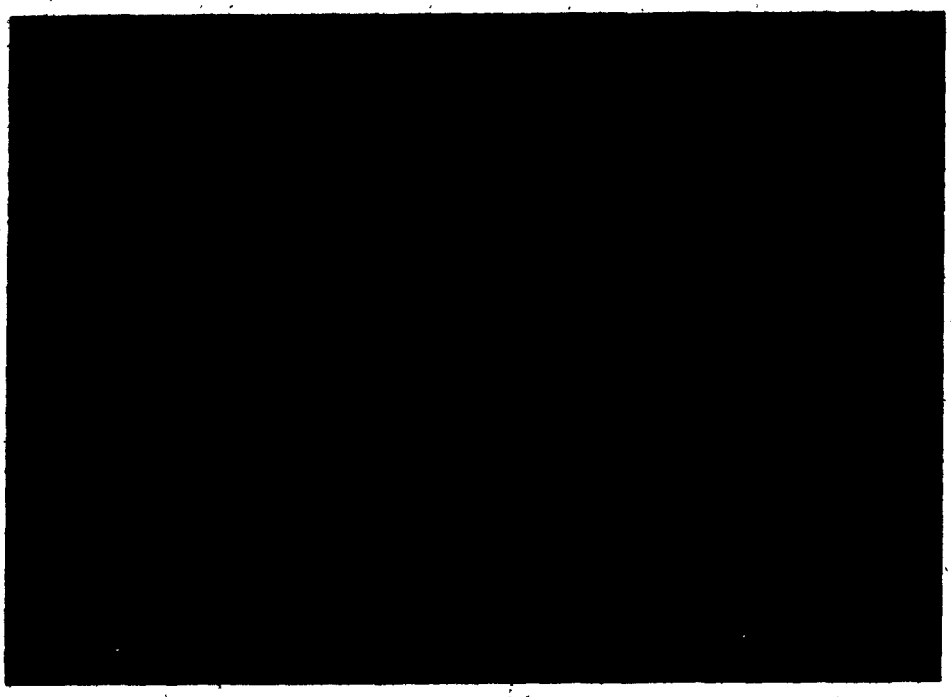


Fig. 8 Comparison of the Constituents of Neutral Peel Extracts of Lemon (A); Grapefruit (B); Orange (C) and of Light Exposed (Ca. 3000 lux) Orange Callus (D) with Authentic Samples of Methylated Flavones (1-5) Chromatographed on Silica Gel in Hexane:butanol (85:15)

1, Sinensetin; 2, Nobiletin; 3, Tetra-O-methylscutellarein; 4, Heptamethoxyflavone; 5, Tangeretin

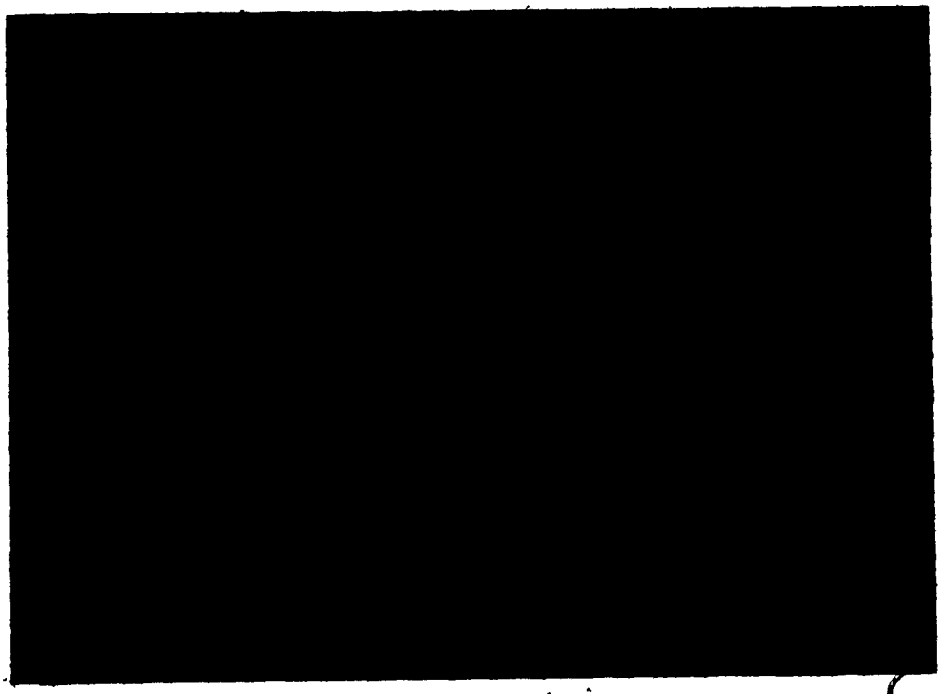


Fig. 9 Comparison of Hydrolyzed Lemon Extracts from Normal Peel (A); Dark Grown Lemon Callus (B) and Light Grown Lemon Callus (C); Unhydrolyzed Extract of Lemon Peel (D); Orange Peel (E); Orange Callus (Dark Grown) (F) and Grapefruit Peel (G), Chromatographed on Alumina Gel with Hexane:butanol (85:15) Followed by a Second Run in Benzene:acetic Acid:water (2:2:1) in the Same Direction.

1, Sinensetin; 2, Nobiletin; 3, Tetra-O-methylscutellarein; 4, Heptamethoxyflavone

Table V: O-Methyltransferase Activity of Calamondin Peel Extracts at Different Stages of Development

Substrates	Immature[1] Calamondin Orange		Mature[2] Calamondin Orange		Ripe[3] Calamondin Orange	
	cpm/g tissue	% Incorp.	cpm/g tissue	% Incorp.	cpm/g tissue	% Incorp.
Quercetin	380	100	12200	100	6080	100
Rhamnetin			11000	90	4600	75
Isorhamnetin			3650	30	1400	23
Kaempferol	0	0	720	6	170	3
Caffeic acid	260	69	2940	24	1920	32
Esculetin			5610	46	1660	27

1 green, 0.5-1.0 cm in diameter
2 green, 1.5-3.0 cm in diameter
3 yellow, 3.0 cm in diameter

D.4 O-Methylation of Flavonoid Compounds by Cell-free Extracts of Citrus and Tobacco

D.4.1 O-Methylation of flavonoids with vicinal ring A hydroxyls

Cell-free extracts of citrus tissues and tobacco cell culture catalyzed O-methylation of a number of substrates having vicinal ring A hydroxyls, albeit with different efficiencies (Table VI). The order of total label incorporation into reaction products was 5,6,7-trihydroxyflavone (baicalein) >> 5,7,8-trihydroxyflavone (norwogonin) > 5,6-dihydroxyflavone > 7,8-dihydroxyflavone.

Both baicalein and 5,6-dihydroxyflavone were O-methylated mainly at position 6, norwogonin at positions 7 and 8, whereas 7,8-dihydroxyflavone was predominantly attacked at position 8.. Monomethylated as well as higher orders of methylated products were observed (Fig. 10 and 11). This methylation pattern correlated with the degree of the negative electron densities for the respective OH-positions (Table VI).

Most of these methylated products were reported to occur naturally in plants (Harborne, 1967; Inagaki et al., 1971).

Table VI: In Vitro O-Methylation of Flavonoid Compounds with Vicinal Ring-A Hydroxyls[1]

Substrate	O-Methyl derivative	Relative Activity (%)[2] Citrus Root	Electron density	OH Position
5,6-Dihydroxyflavone	5,6-	35[3]		
	6-	50	-0.1386	6
	5-	5	-0.0865	5
		10		
7,8-Dihydroxyflavone	7,8-	10[4]		
	8-	65	-0.0369	8
	7-	5	+0.0542	7
		20		
5,6,7-Trihydroxyflavone (Baicalein)	5,6-/6,7-/5,6,7-	30[5]		
	6-	40	-0.2326	6
	5-/7-	6	-0.1114	7
		25	-0.1409	5
5,7,8-Trihydroxyflavone (Norwogonin)	7,8-	35[6]	-0.2089	8
	8-/7-	45	-0.0819	7
		20	+0.1843	5

[1] Standard enzyme assay was used as described in the Methods Section

[2]* After separation of individual products by TLC, spots were scraped off the plate and their activities determined by LSC. Total cpms in products 5-8

[3] 7000 cpm; [4] 9000 cpm; [5] 10500 cpm; [6] 6000 cpm

* Detailed Table VI in Appendix Section I

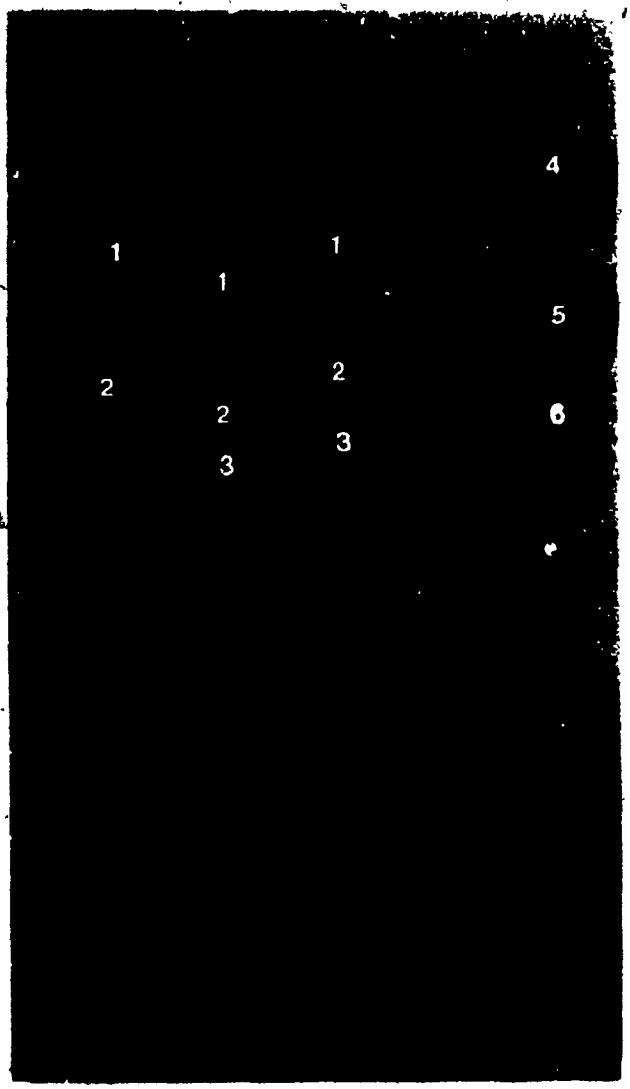


Fig. 10 Radioautograph Showing the Methylated Products of 5,6,7-Trihydroxyflavone (Baicalein) and 5,6,-Dihydroxyflavone

A-C Products of 5,6,7-trihydroxyflavone A: Tobacco cell extract. B: Citrus root extract. C: Citrus peel extract. (1) 5,6-/6,7-dimethylether; (2) 6-monomethylether; (3) 5-/7-monomethylether. D-E: Products of 5,6-dihydroxyflavone D: Tobacco cell extract. E: Citrus root extract. (4) 5,6-dimethylether; (5) 6-monomethylether; (6) 5-monomethylether.

Fig. 11 Radioautograph Showing the Methylated Products of 7,8-Dihydroxyflavone and 5,7,8-Trihydroxyflavone (Norwogonin)

A-C Citrus Root Extract A: Products of 7,8-dihydroxyflavone (1) 7,8-dimethylether; (2) 8-monomethylether; (3) 7-monomethylether. Enzyme control. B: Products of 5,7,8-trihydroxyflavone: (1) 7,8-dimethylether; (2) 7-/8-monomethylether. C: 2-Dimensional separation of methylated products of 5,7,8-trihydroxyflavone: (1) 7,8-dimethylether; (2) 7-monomethylether; (3) 8-monomethylether.

D.4.2 O-Methylation of flavonoids with vicinal ring A hydroxyls and ring B substitution

Seven substrates have been tested for their methyl acceptor ability using citrus and tobacco cell extracts. The order of label incorporation was as follows: 6,7-dihydroxy-4'-methoxyisoflavone (texasin) >> 5,6,7,4'-tetrahydroxyflavone (scutellarein) > 6,7,4'-trihydroxyisoflavone > 3,5,7,8,3',4'-hexahydroxyflavone (gossypetin) = 5,7,8,4'-tetrahydroxyflavone (8-hydroxyapigenin) > 3,5,6,7,3',4'-hexahydroxyflavone (quercetagetin) > 5,7,8-trihydroxy-4'-methoxyflavone (8-hydroxyacacetin).

Reaction products that were isolated showed higher amount of label in monomethylated than in di- and trimethyl products (Fig. 12-15; Table VII). Both texasin and 6,7,4'-trihydroxyisoflavone were methylated almost predominantly at position 6, but not to the exclusion of the 7 position. Scutellarein gave the 4'-methyl and 6,4'-dimethyl derivatives, indicating meta and para O-methylation of ring A and ring B hydroxyls, respectively. 5,7,8,4'-Tetrahydroxyflavone was methylated at positions 8 and 4', whereas 5,7,8-trihydroxy-4'-methoxyflavone was methylated at positions 7 and 8, indicating both meta and para directed reactions. Quercetagetin and gossypetin underwent stepwise methylation, thus quercetagetin yielded the following products: 3,7-dimethyl; 3,6,7-trimethyl; 3,6,7,4'-tetramethyl and 3,6,7,3'-tetramethyl, whereas gossypetin produced three reaction products whose identity could not be established owing to lack of reference compounds.

Fig. 12. Radioautograph Showing the Methylated Products of 6,7,4'-Trihydroxyisoflavone and 6,7-Dihydroxy-4'-methoxyisoflavone (Texasin)

A-B. Citrus Root Extract. A:- Products of 6,7,4'-trihydroxyisoflavone. (1) 6,7,4'-trimethylether; (2) 6,4'/7,4'-dimethylether; (3) 6-monomethylether; (4) 4'-monomethylether. B: Products of 6,7-dihydroxy-4'-methoxyisoflavone. (1) 6,7,4'-trimethylether; (2) 6,4'-/7,4'-dimethylether

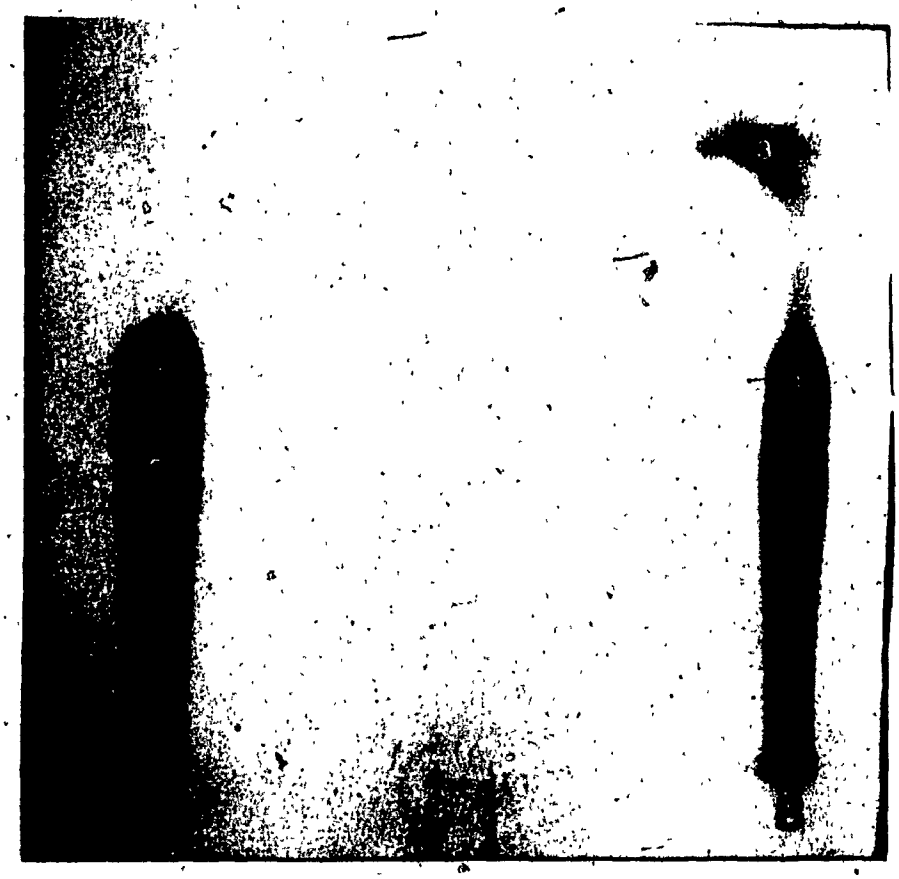


Fig. 13 Two-dimensional Radioautograph Showing the Products of 5,6,7,4'-Tetrahydroxyflavone (Scutellarein)

A: Citrus root extract. B: Tobacco cell extract:
(1) 6,4'-dimethylether; (2) 4'-/6-monomethylether; (3) trimethylether

Fig. 14 Radioautograph Showing the Methylated Products of 5,7,8,4'-Tetrahydroxyflavone

Citrus root extract: (1) 8,4'-dimethylether; (2) 4'-monomethylether

Fig. 15 Radioautograph Showing the Methylated Products of 3,5,6,7,3',4'-Hexahydroxyflavone (Quercetagetin) and 3,5,7,8,3',4'-Hexahydroxyflavone (Gossypetin)

A-C: Tobacco cell extract. D-F: Citrus root extract. G-H: Citrus peel extract. A,B,D,E and G: reaction products of 3,5,6,7,3',4'-hexahydroxyflavone. C,F and H: reaction products of 3,5,7,8,3',4'-hexahydroxyflavone. E: (1) penta or hexamethylether; (2) 3,6,7,3'-tetramethylether; (3) 3,6,7,4'-tetramethylether; (4) 3,6,7-trimethylether; (5) di-/trimethylether; (6) 3,7-dimethylether. C: unknown di-,tri- and tetramethylethers of 3,5,7,8,3',4'-hexahydroxyflavone

Table VII: In Vitro O-Methylation of Flavonoids with Vicinal Ring-A Hydroxyls and B-Ring Substitution[1]

Substrate	O-Methyl derivative	Relative Activity (%)[2] Citrus Root	Electron density	OH Position
6,7,4'-Trihydroxy-isoflavone	6,7,4'-	10[3]		
	6,4'-/7,4'-	10	-0.0985	6-
	6-/7-	50	-0.0360	7-
	4'-	15	-0.0235	4'-
		15		
6,7-Dihydroxy-4'-methoxyiso-flavone (Texasin)	Di/Trimethyl	70[4]	-0.1390	6-
	6,4'/7,4'	10	-0.1066	7-
		20		
5,6,7,4'- Tetra-hydroxyflavone (Scutellarein)	6,4'-	60[5]	-0.0213	6-
	4'-/6-	30	+0.0984	7-
		10	+0.1347	5-
			-0.1395	4'-
5,7,8,4'- Tetra-hydroxyflavone (8-Hydroxyapigenin)	8,4'-	37[6]	-0.2495	8-
	4'-	31	+0.0991	7-
		32	+0.2201	5-
			-0.1519	4'-
5,7,8-Trihydroxy-4'-methoxyflavone (8-Hydroxyacacetin)	8,4'-/Tri-OCH_3	60[7]	-0.2089	8-
	7,4'-	2	-0.0819	7-
		38	+0.1843	5-
3,5,6,7,3'4'-Hexahydroxyflavone (Quercetagetin)	Penta-/Hexa	5[8]	-0.2969	3-
	3,6,7,3'-	25	+0.0821	6-
	3,6,7,4'-	20	+0.0032	7-
	3,6,3'-/3,6,7-	10	+0.0846	5-
	Di-/Tri-	trace	-0.1086	3'-
	3,7-	trace	-0.2079	4'-
3,5,7,8,3',4'-Hexahydroxyflavone (Gossypetin)	Tetra-/Tri-OCH_3	15[9]	-0.2956	3-
	Di-/Tri-OCH_3	15	-0.1059	8-
	Di-OCH_3	10	-0.1162	7-
		60	-0.2051	5-
			-0.0537	3'-
			+0.1052	4'-

Footnotes for Table VII

[1] Standard enzyme assay was used as described in the Methods Section

[2]* After separation of individual products by TLC, spots were scraped off the plate and their activities determined by LSC. Total cpms in products 5-19

[3] 12000 cpm; [4] 7000 cpm; [5] 6000 cpm; [6] 4000 cpm; [7] 800 cpm; [8] 2500 cpm; [9] 2000 cpm

* Detailed Table VII in Appendix Section I

The methyl derivatives of the following compounds used have been reported in several plant species: texasin (Wong, 1975); Sccutellarein, (Swift, 1967; Wong, 1975); 5,7,8-trihydroxy-4'-methoxyflavone (Tatum and Berry, 1972); quercetagetin (Bohm and Collins, 1979); gossypetin (Tatum and Berry, 1972; 1978).

D.4.3 O-methylation of flavonoids without vicinal ring A hydroxyls

Seven flavonoids were tested as methyl acceptors and their total methyl incorporation was as follows: 5,7,3',4'-tetrahydroxy-flavone (luteolin) >> 3,5,7-trihydroxyflavone (galangin) > 5,7,4'-trihydroxyflavone (apigenin) > 3,5,7,4'-tetrahydroxyflavone (kaempferol) > 5,7,4'-trihydroxy-3'-methoxyflavone (chrysoeriol) 5,7-dihydroxy-4'-methoxyflavone.

Most of the substrates with ring B hydroxyls were methylated at the para position of that ring (Table VIII; Fig. 16). Therefore, apigenin yielded the 4'-monomethyether (acacetin), whereas kaempferol was methylated predominantly at the 4'- and 7- positions by the root and peel enzymes, respectively. Luteolin was predominantly methylated at the 3'- position with lower incorporation at 4'. Only luteolin yielded a small amount of a dimethylated product. The B-ring substituted substrates, acacetin and chrysoeriol, did not undergo further methylation.

The methyl acceptor ability of substrates without ring B substitution (chrysin and galangin) showed that chrysin was not methylated by any of the cell free extracts used, whereas the methylation of galangin yielded a product that cochromatographed with a

Table VIII: In Vitro O-Methylation of Flavonoid Compounds Without Vicinal Ring-A Hydroxyls[1]

Substrate	O-Methyl derivative	Relative Activity (%)[2] Citrus Root	Electron density	OH Position
5,7-Dihydroxyflavone (Chrysin)		SHOWED NO ACTIVITY		
3,5,7-Trihydroxyflavone (Galangin)	3,7-	5[3]	-0.3273	3
	3-	70	+0.0684	5
	7-	10	-0.0127	7
	unidentified	15		
5,7,4-Trihydroxyflavone (Apigenin)	7,4'-	1[4]		
	4'-	90	+0.0349	7
	unidentified	9	-0.1903	4'
5,7-Dihydroxy-4'-methoxyflavone (Acacetin)	7,4'-	Trace		
	unidentified	-		
5,7,3',4'-Tetrahydroxy-flavone (Luteolin)	7,3'-	5[5]	+0.0293	5
	7,4'-	2	+0.0242	7
	3'-	75	-0.1217	3'
	4'-	6	-0.2176	4'
5,7,4-Trihydroxy-3'-methoxyflavone (Chrysoeriol)	7,3'-	Trace	+0.0069	5
	3'-/3',4'	Trace	+0.0416	7
			-0.1930	4'
3,5,7,4'-Tetrahydroxy-flavone (Kaempferol)	7,4'-	Trace[6]		
	4'-	40	0.3384	3
	7-	10	0.3334	5
	3-	20	0.0028	7
		20	+0.1537	4
	unidentified	10		

Footnotes for Table VIII

[1] Standard enzyme assay was used as described in the Methods Section

[2]* After separation of individual products by TLC, spots were scraped off the plate and their activities determined by LSC. Total cpms in products 5-19

[3] 5000 cpm; [4] 1500 cpm; [5] 10000 cpm; [6] 15000 cpm

*. Detailed Table VIII in Appendix Section I

Fig. 16 Radioautograph Showing the Methylated Products of 3,5,7-Trihydroxyflavone (Galangin); 5,7,4'-Trihydroxyflavone (Apigenin); 3,5,7,4'-Tetrahydroxyflavone (Kaempferol) and 5,7,3',4'-Tetrahydroxyflavone (Luteolin)

A,B,C,E: Citrus root extract. D: Citrus peel ext. A: Reaction products of 3,5,7-trihydroxyflavone: (1) 3,7-dimethylether; (2) 3-monomethylether; (3) 7-monomethylether. B: Reaction products of 5,7,4'-trihydroxyflavone: (1) 7,4'-dimethylether; (2) 4'-monomethylether. C: Reaction products of 3,5,7,4'-Tetrahydroxyflavone: (1) dimethylether; (2) 4'-monomethylether; (3) 7-monomethylether. (4) 3-monomethylether. D-E: Reaction products of 5,7,3',4'-tetrahydroxyflavone: (1) 7,3'-dimethylether; (2) 7,4'-dimethylether; (3) 3'-monomethylether; (4) 4'-monomethylether.

sample of 3-methylgalangin, thus demonstrating for the first time the existence of a 3-O-methyltransferase in citrus and tobacco tissues.

Although kaempferol has been reported to occur in citrus (Kefford and Chandler, 1970) there is no report, as yet, on the occurrence of its methylethers in this tissue. However, they have been reported in several other plant species (Gottlieb, 1975).

D.4.4 Sequential O-methylation of quercetin and its derivatives

Cell free extracts of citrus peel, root, callus and tobacco suspension culture catalysed the O-methylation of quercetin and a number of its methyl ethers in a stepwise fashion (Table IX, Fig. 17-19) The efficiency of substrates as methyl acceptors, in descending order, was as follows: 3-methylquercetin > 3,5,7,3',4'-pentahydroxyflavone (quercetin) > 3,5,7,3'-tetrahydroxy-4'-methoxyflavone (tamarixetin) > 3,5,3',4'-tetrahydroxy-7-methoxyflavone (rhamnetin) > 3,5,7,4'-tetrahydroxy-3'-methoxyflavone (isorhamnetin) > 3,5,3'-trihydroxy-7,4'-dimethoxy flavone (ombuin) > 3',4'-dimethylquercetin.

The above substrates were further methylated to yield a higher order of their methyl derivatives. Thus, quercetin yielded the following methyl ethers: 3-; 7-/4'-; 3'-; 3,7-; 7,3'-/7,4'-; 3,3'-/3,4'-; 3,7,4'- and traces of 3,7,3'4'- methyl derivatives (Fig. 17 and 18). 3-Methylquercetin and 4'-methylquercetin (tamarixetin) were transformed to the dimethyl ethers 3,7-; 3,3'-/3,4'- and 7,4'-; 3,4'-, respectively (Fig. 19). Two other

monomethyl ethers of quercetin, 7-methylquercetin (rhamnetin) and 3'-methylquercetin (isorhamnetin) gave rise to dimethyl-(7,3'-/7,4'-); trimethyl-(3,7,3'-; 3,7,4'-) and tetramethyl-(3,7,3',4') derivatives (Fig. 17). The 7,4'-dimethyl ether, ombuin, was further methylated to the 3,7,4'-trimethyl- and a trace amount of the 3,7,3',4'-tetramethyl ethers (Fig. 19), whereas 3',4'-dimethylquercetin yielded a number of methylated products, two of which were tentatively identified as 3,3',4'-trimethyl- and 7,3',4'-trimethylquercetin (Fig. 19). The identification of the 3,3'- and 3,4'-methylethers of quercetin is also tentative, based on comparison of their Rf values with those published (Jay et al., 1975).

Although quercetin is widely distributed in the plant kingdom, there has been no report on the occurrence of its methylated derivatives in citrus, with the exception of isorhamnetin (Kefford and Chandler, 1970). However the 7-methylether (rhamnetin), 3'-methylether (isorhamnetin), 4'-methylether (tamarixetin) and the 7,3'-dimethylether (rhamnazin) as well as the 7,4'-dimethylether (ombuin) have been isolated from a variety of plants (Harborne et al., 1975; Wollenweber and Dietz, 1981). The 3,7,4'-trimethylether (ayanin) has also been reported (King, et al., 1952; Harborne, 1967). The 3-monomethyl and 3,3'-dimethyl ethers were isolated from the flowers of tobacco by Yang et al (1960).

Table IX: In Vitro O-Methylation of Flavonoid Compounds of the Quercetin Series[1]

Substrate	O-Methyl derivative	Relative Activity (%)[2] Citrus Root	Electron density	OH. Position
3,5,7,3',4'-Pentahydroxyflavone (Quercetin)	7,3'-/7,4'-	31[3]	-0.3090	3
	3,7-/3,3',4'-	2	+0.0474	5
	3'-/3,3'-/3,4'-	16	-0.0162	7
	7-/4'-	9	-0.1100	3'
	3-	3	+0.2089	4'
	5-	10		
	Unidentified	29		
5,7,3',4'-Tetrahydroxy-3-methoxyflavone (3-Methylquercetin)	3,7-	65[4]	-0.0287	5
	3,3'-/3,4'-	30	-0.0002	7
	Unidentified	5	+0.0747	3'
			0.1578	4'
3,5,3',4'-Tetrahydroxy-7-methoxyflavone (Rhamnetin)	Tri-Tetra-OCH_3	trace[5]	-0.3093	3
	3,7,4'-/3,7,3'-	17	-0.0477	5
	7,4'-/7,3'-	41	+0.1092	3'
	3,7-	16	-0.2079	4'
	3'-	3		
	7-	5		
	Unidentified	18		
3,5,7,4'-Tetrahydroxy-3'-methoxyflavone (Isorhamnetin)	Tri-/Tetra-OCH_3	trace[6]	-0.3240	3
	7,3'-	17	+0.0328	5
	3,3',4'-	5	-0.0037	7
	3'-/3,3'-	8	-0.1768	4'
	7-	10		
	Unidentified	60		
3,5,7,3'-Tetrahydroxy-4'-methoxyflavone (Tamarixetin)	7,4'-	75[7]	+0.3262	3
	3,4'-	4	-0.0326	5
	Unidentifed	21	+0.0033	7
			+0.0934	3'
3,5,3'-Trihydroxy-4'-dimethoxyflavone (Ombuin)	3,7,3',4'-	1[8]		
	3,7,4'-		+0.3258	3
	7,4'-/7,3',4'-		-0.0327	5
	Unidentified		-0.0932	3'
3,5,7-Trihydroxy-3',4'-dimethoxyflavone (3',4'-dimethyl-quercetin)	7,3',4'-	43[9]	-0.3080	3
	Internal substr.	2	+0.0358	5
	3,3',4'-	7	+0.0053	7
	3',4'-	33		
	Unidentified	17		

Footnotes for Table IX

[1] Standard enzyme assay was used as described in the Methods Section

[2]* After separation of individual products by TLC, spots were scraped off the plate and their activities determined by LSC. Total cpms in products 5-19

[3] 16500 cpm; [4] 10000 cpm; [5] 21000 cpm; [6] 12500 cpm; [7] 23250 cpm; [8] 20250 cpm; [9] 10750 cpm;

* Detailed Table IX in Appendix Section I

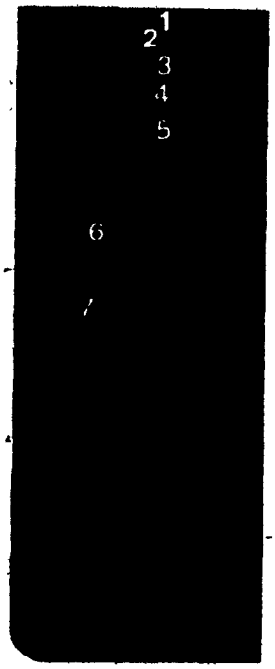


Fig. 17 Radioautograph Showing the Methylated Products of A: 3,5,7,3',4'-Pentahydroxyflavone (Quercetin); B:3,5,3',4'-Tetrahydroxy-7-methoxyflavone (Rhamnetin) and C: 3,5,7,4'-Tetrahydroxy-3'-methoxyflavone (Isorhamnetin). (Citrus Root Extract).

A: Quercetin. (1) 3,7,3',4'-tetramethylether; (2) 3,7,4'-/3,7,3'-trimethylether; (3) 7,4'-/7,3'-dimethylether; (4) 3'-mono/3,3'-/3,4'--/3,7-dimethylether; (5) 7-/4'-monomethylether; (6) 3-monomethylether; (7) 5-monomethylether. B: Rhamnetin. (1) 3,7,3',4'-tetramethylether; (2) 3,7,4'-/3,7,3'-trimethylether; (3) 7,4'-/7,3'-dimethylether; (4) 3,7-dimethylether; (5) (7-/4'-monomethylether. C: Isorhamnetin. (1) 3,7,3',4'-tetramethylether; (2) 3,7,3'-trimethylether; (3) 7,3'-dimethylether; (4) 3'-/3,3'-dimethylether; (5) monomethylether.

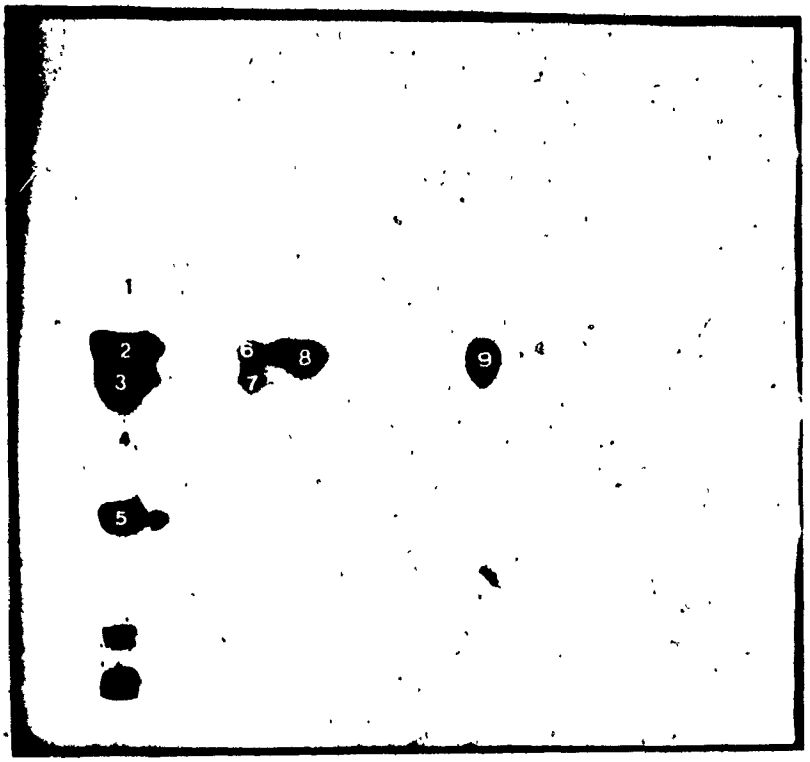


Fig. 18 Two-dimensional Radioautograph Showing the Methylated Products of Quercetin (Citrus Peel Enzyme).

(1) 7,4'-/7,3'-dimethylether (Ombuin). (2) 3'-dimethylether (Isorhamnetin). (3) 7-/4'-monomethylether (Rhamnetin/Tamarixetin). (4) 3-monomethylether. (5) 5-monomethylether. (6,7,9) unidentified products, possibly 3,3'-/3,4'- and product(s) having position 5 methylated. (8) 3,7-dimethylether.

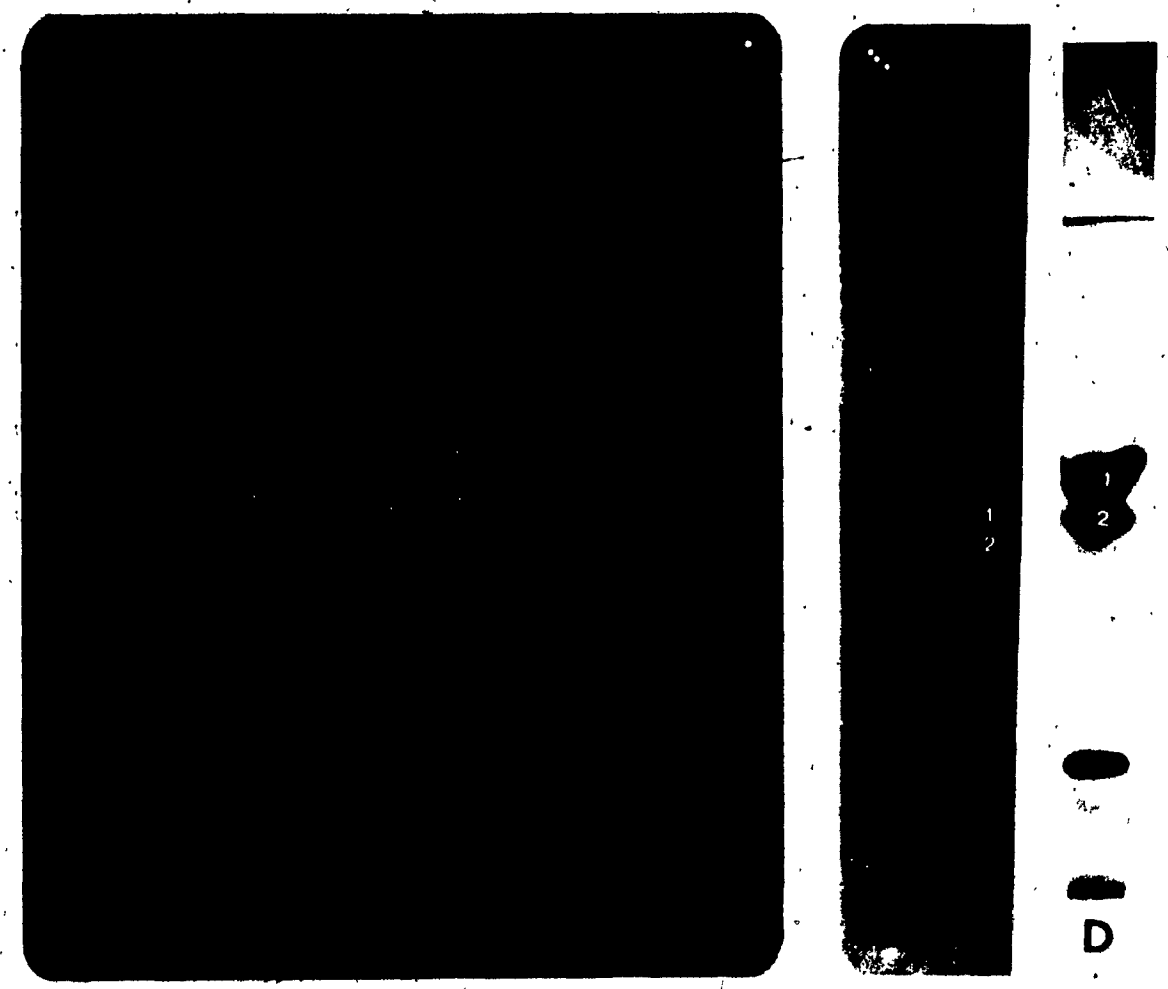


Fig. 19 Radioautograph Showing the Various Methylation Products of Quercetin and its Analogues. A: 3,5,7,3',4'-Pentahydroxy-flavone (Quercetin). B: 3,5,3',4'-Tetrahydroxy-7-methoxy-flavone (Rhamnetin). C: 3,5,7,4'-Tetrahydroxy-3'-methoxy-flavone (Isorhamnetin). D: 3,5,7,3'-Tetrahydroxy-4'-methoxy-flavone (Tamarixetin). E: 3,5,3'- Trihydroxy-7,4'-dimethoxy-flavone (Ombuin). F: 3,5,7,-Trihydroxy-3',4'-dimethoxy-flavone. H: 5,7,3',4'-Tetrahydroxy-3-methoxyflavone. (Citrus Root Extract)

Fig. 19 (continued)

A (1) 7,4'-/7,3'-dimethylether. (2) 3'-monomethylether.
(3) 7-/4'-monomethylether.

B (1) 3,7,4'-trimethylether (2) 7,4'-/7,3'-dimethylether.
(3) 3,7-dimethylether.

C (1) 7,3'-dimethylether.

D (1) 7,4'-dimethylether. (2) 3,4'-dimethylether.

E (1) 3,7,3',4'-tetramethylether. (2) 3,7,4'-trimethylether.
(3) Band coinciding with substrate.

F (1) 7,3',4'-trimethylether? (2) 3,3',4'-trimethylether?
(3) Band coinciding with substrate.

G Enzyme extract internal substrates (control).

H (1) 3,7-dimethylether. (2) 3,~~3'-/3~~,4'-dimethylether.

D.5 Discussion

D.5.1 Callus growth and flavonoid synthesis

The results presented here demonstrate the initiation and continued growth of callus tissue originating from mature orange flavedo. The relative difficulty encountered in callus initiation on orange peel, as compared with other tissue explants, may be due to the release of certain flavonoid or volatile constituents during excision of the tissue. Such compounds have been reported to inhibit cell de-differentiation and callus formation (Bagni and Fracassini 1966).

Illumination of the cultures was found to stimulate both cell division and tissue differentiation, which resulted in lignification (Fig. 6B) and flavonoid production (Table IV). The dark grown tissue, on the other hand, was characterized by a high degree of cell uniformity and the lack of either cell differentiation or flavonoid formation (Fig. 9F). These striking differences appear to be closely correlated with the high activity of phenylalanineammonia-lyase (PAL) in the light grown tissue (Fig. 22), a key enzyme of phenolic synthesis which is widely distributed in plants (Neish, Young et al., 1966, 1964). Light induction of PAL in intact tissues (Zucker, 1965) and tissue cultures (Nitsch and Nitsch, 1966; Thorpe et al., 1971) has been well documented, and its stimulation of flavonoid synthesis (Amrhein and Zenk, 1971; Hahlbrock and Wellmann, 1970) and lignin formation (Higuchi and Barnaud, 1966) has been reported.

Although PAL has been shown to occur in grapefruit albedo callus, its activity was thought to be light-independent (Thorpe et al, 1971). This is in contrast with the observations reported here with orange peel callus (Brunet and Ibrahim, 1973), where PAL activity is light dependent and attains its maximum activity after two days in culture (Fig. 22). This demonstrates the requirement for a high level of PAL activity prior to tissue differentiation and flavonoid synthesis in orange tissue culture.

Comparison of the flavonoid content of both orange peel and its callus tissue (Table IV) shows that the amount of each flavone formed in the callus was relatively low, as compared with that in the peel tissue. However, it is interesting to note that the biosynthetic mechanism for the formation of polymethylated flavones functions in callus tissues, and that sinensetin and nobiletin are among the major components of both callus and peel tissues.

Light requirement for OMT activity or enzyme induction appears to be similar to that for PAL, since calli maintained in the dark exhibited very little O-methylating activity. This is in agreement with the earlier observation on the extremely low amounts of polymethylated flavones in dark grown calli (Fig. 8). A direct relationship seems to exist between OMT activity and fruit development (Table V). Immature fruits exhibited low enzyme activity which increased with fruit development reaching a maximum before the onset of yellowing, then decreased steadily thereafter. These results correlate well with the seasonal variations of methylated flavones observed in orange peel (Swift, 1967).

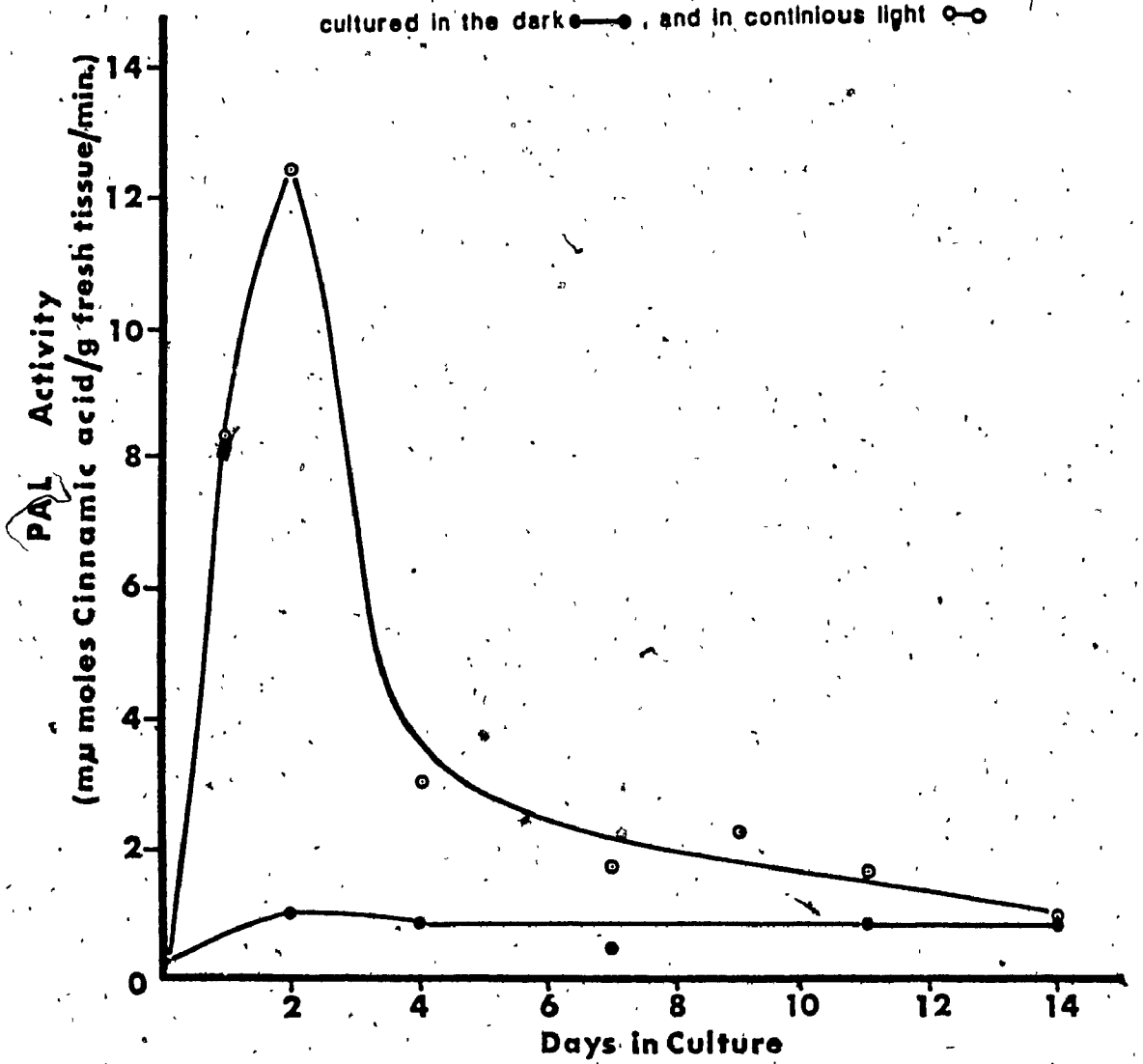


Fig. 22 Phenylalanine ammonia -lyase activity of orange callus tissue

It has often been debated whether morphogenesis prescribes the pattern of secondary metabolite synthesis, or whether the latter is in fact the determinant of the process of morphogenesis. In the experimental circumstances reported here, it may be a coincidence that the conditions that led to tissue differentiation of orange callus also resulted in flavonoid production. Nevertheless, flax cotyledonary tissue was reported to lose its potential for flavonoid production as soon as it was cultured *in vitro*, although its callus attained a high degree of cellular differentiation (Liau and Ibrahim, 1973), suggesting that morphogenesis and secondary metabolite synthesis may not necessarily be interrelated as is often claimed.

D.5.2 Comparison of *O*-methyltransferase activity

The results obtained with the various cell-free extracts and substrates used, clearly indicate that ring-A methylation occurs in all the species tested, and suggest that *O*-methylation is a fairly common reaction involving *ortho*-, *meta*- and *para*-*O*-methylation.

The optimum pH for *O*-methylation of flavonoids in citrus tissue was found to be 7.5 (Fig. 23). There was a reduction in the extent of methylation at higher (pH 8.5) or lower (pH 6.5) values. This may be explained by the fact that increasing pH would lead to quinoid formation, whereas lowering the pH could affect the 2-3 double bond and the carbonyl group of ring C (protonation effect). There are, however, some instances where optimal methylation was reported to proceed at a pH as low as 6.5 (Poulton and Butt, 1975; Poulton *et al.*, 1976b) and as high as 9.6-9.8 (Ebel *et al.*, 1972).

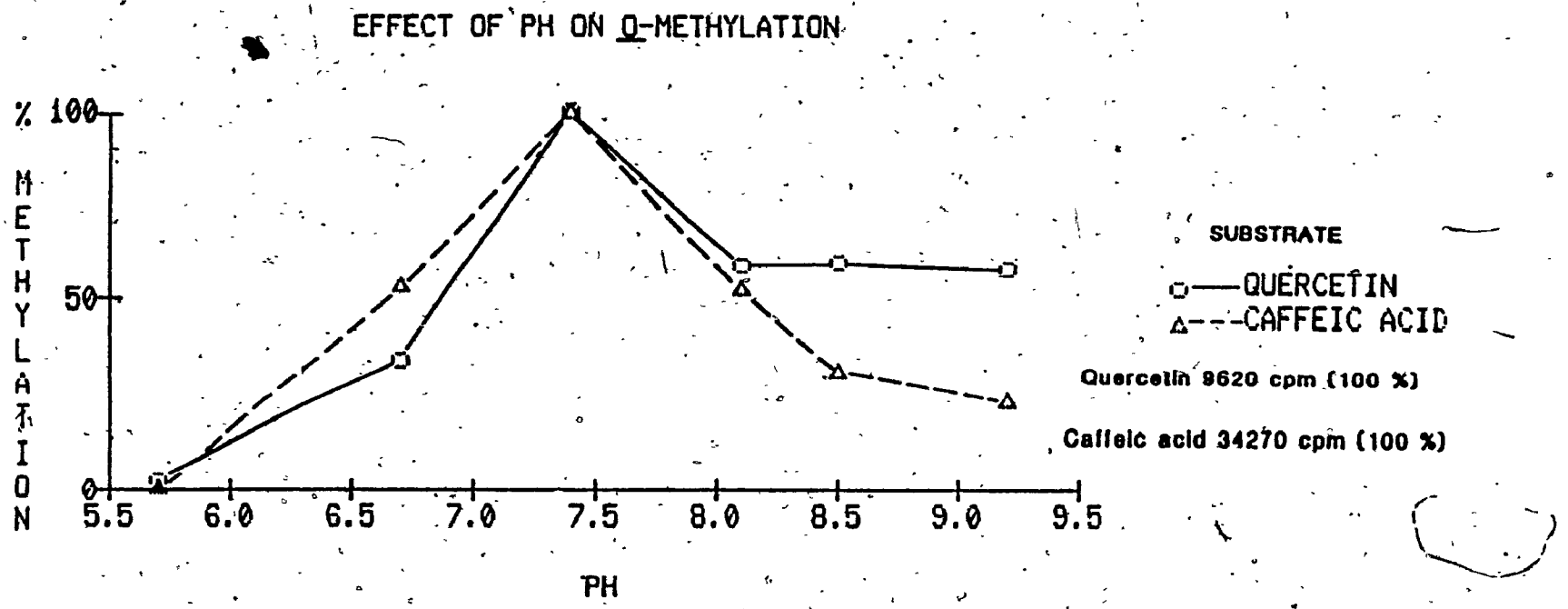


Fig.23 Effect of pH on O-methylation

Flavonoids with vicinal hydroxyl groups at positions 5-, 6-, 7- or 8- and lacking ring B substitution exhibited the highest O-methylation as well as high electron density for positions 6 and 8, except for 7,8-dihydroxyflavone where position 7 had a slightly higher electron density (Table VI). On the other hand, O-methylation of flavonoid compounds with vicinal ring A hydroxyls and B-ring substitution indicated that compounds with the 6,7,4'- or 5,6,7,4'-configuration were more efficiently methylated than those having the 5,7,8,4'-configuration. The influence of B-ring substituion on A-ring methylation was difficult to assess with the limited number of substrates available. Introduction of a methyl group at the 4'- position of 5,7,8,4'-tetrahydroxyflavone reduced its ability to undergo further methylation, whereas it increased the reactivity of 6,7-dihydroxy-4'-methoxyisoflavone (texasin). The higher methyl incorporation by the isoflavone may be due either to the greater reactivity of the hydroxyls at positions 6 and 7 as determined by electron density calculations, or a greater enzyme affinity for the 6,7-configuration as opposed to the 5,7,8-structure. This remains to be investigated with a more purified enzyme.

O-Methylation of flavonoids without vicinal ring A hydroxyls showed that chrysin (5,7-dihydroxyflavone) was not methylated by any of the cell-free extracts used, whereas galangin (3,5,7-trihydroxyflavone) proved to be a reasonably good methyl acceptor and was predominantly methylated at the 3-position; thus establishing, for the first time, the existence of a 3-O-methyltransferase in citrus root tissue. The

fact that the enzyme preparation from tobacco cell culture also catalysed 3-methylation of both galangin and quercetin, supports the original observation and provides a good source for the purification of this enzyme. Recently, 3-O-methyltransferase has been purified from Crysosplenium americanum and its specificity for the 3-position of quercetin was established (De Luca and Ibrahim, 1982).

Other flavonoid compounds were very poor methyl acceptors, except for luteolin which was predominantly methylated on ring B. Electron density calculations suggested reactivity for position 3 of galangin and kaempferol, position 4' of apigenin, and positions 3' and 4' of luteolin. The poor methyl acceptor abilities of apigenin and kaempferol, however, seems to indicate that the citrus and tobacco enzymes have no affinity for these substrates, and may possibly be due to para-quinoid formation at the 4'-position, resulting in rigid structure and concomitant resonance of the flavonoid ring system.

The results of methylation of quercetin and its analogs, lead to some interesting speculation. The two major monomethyl products formed from quercetin were isorhamnetin (3'-MeQ) and rhamnetin (7-MeQ). Further methylation of either of the latter compounds indicated that rhamnetin was a better substrate than isorhamnetin (Table IX). Isorhamnetin, on the other hand, produced relatively smaller amounts of the dimethyl product (7,3'-diMeQ) and a trace of the trimethyl product. This seems to indicate that rhamnetin is a major intermediate in the methylation sequence and that methylation at the 7-position is a key step in the formation of partially methylated

products. It is interesting to note that further methylation of 3-methylquercetin gave rise to two dimethyl derivatives (3,7'- and 3,3'-/3,4'-) but none of the higher orders of methyl derivatives. This work has shown the methylation of almost all positions on the flavonoid ring system. Although it is difficult to demonstrate the biosynthesis of fully methylated flavones in vitro, however, it seems possible that partially methylated intermediates may undergo hydroxylation at positions 6 and/or 8 and further methylation, to give rise to the polymethylated flavones known to occur in Chrysosplenium (Collins et al., 1981) and citrus (Swift, 1967). The results obtained with scutellarein, quercetagetin and gossypetin indicate that the biogenesis of the polymethylated flavones of citrus (Tatum and Berry, 1978) is unlikely the result of methylation of the hydroxy analogs, but rather by an alternating process of hydroxylation and methylation of partially methylated intermediates. A proposed scheme of stepwise methylation of quercetin is shown in Figure 24. However, final proof of these methylation steps must await purification of the enzymes involved from a suitable source, since all attempts to isolate and purify the enzymes from calamondin were unsuccessful.

Extremely low or almost no methylation was observed with the flavonol glycosides used as substrates, indicating that glycosylation is a later step in the biosynthesis of flavonoids (Hahlbrook and Grisebach, 1975; Grisebach, 1977). Very recently, the methylation of anthocyanins in Petunia (Jonsson et al., 1982) and of vitexin 2"-O-rhamnoside in Avena sativa (Knogge and Weissenböck, 1984) have

been reported, suggesting that the order of methylation and glycosylation may vary in different tissues.

The results reported here suggest the existence of a number of OMT's in calamondin cell free extracts that catalyze the O-methylation of almost all hydroxyl groups of the flavonoid ring system, including both rings A and B. Whether citrus enzymes are flavonoid-specific remains to be elucidated, since a number of other non-flavonoid substrates such as caffeic acid (3,4-dihydroxycinnamic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), catechol (1,2-dihydroxybenzene and esculetin (6,7-dihydroxy-2H-1-benzopyran-2-one) were methylated to their respective meta and para O-methyl derivative, (results not shown), in a manner similar to that of the yeast enzymes (Müller-Enoch, et al., 1976). Furthermore, it is possible that there exists in citrus meta and para specific enzymes for both rings A and B of flavonoid compounds. Recently, DeLuca and Ibrahim (1982) reported the purification and properties of four flavonol-specific O-methyltransferases from C. americanum, which were specific for positions 3, 6, 7 a[illegible]4' (DeLuca & Ibrahim, 1985). Similarly, the methylation of pos[illegible]s 8 and 3' in Lotus corniculatus was shown to be catalysed by two distinct enzymes (Jay et al., 1983).

The OMT system of citrus appears to be distinct from those previously reported which catalyse single methylation steps (Ebel et al., 1972; Wengenmeyer et al., 1974; Jonsson et al., 1982; Knogge and Weissenböck, 1984) and from that of C. americanum which catalyses an orderly sequence of methylation steps (Q → 3Q → 3,7Q → 3,7,4'Q).

Citrus OMT system mediated single methylation steps at different positions as well as stepwise methylation of partially methylated intermediates as shown in Fig. 24.

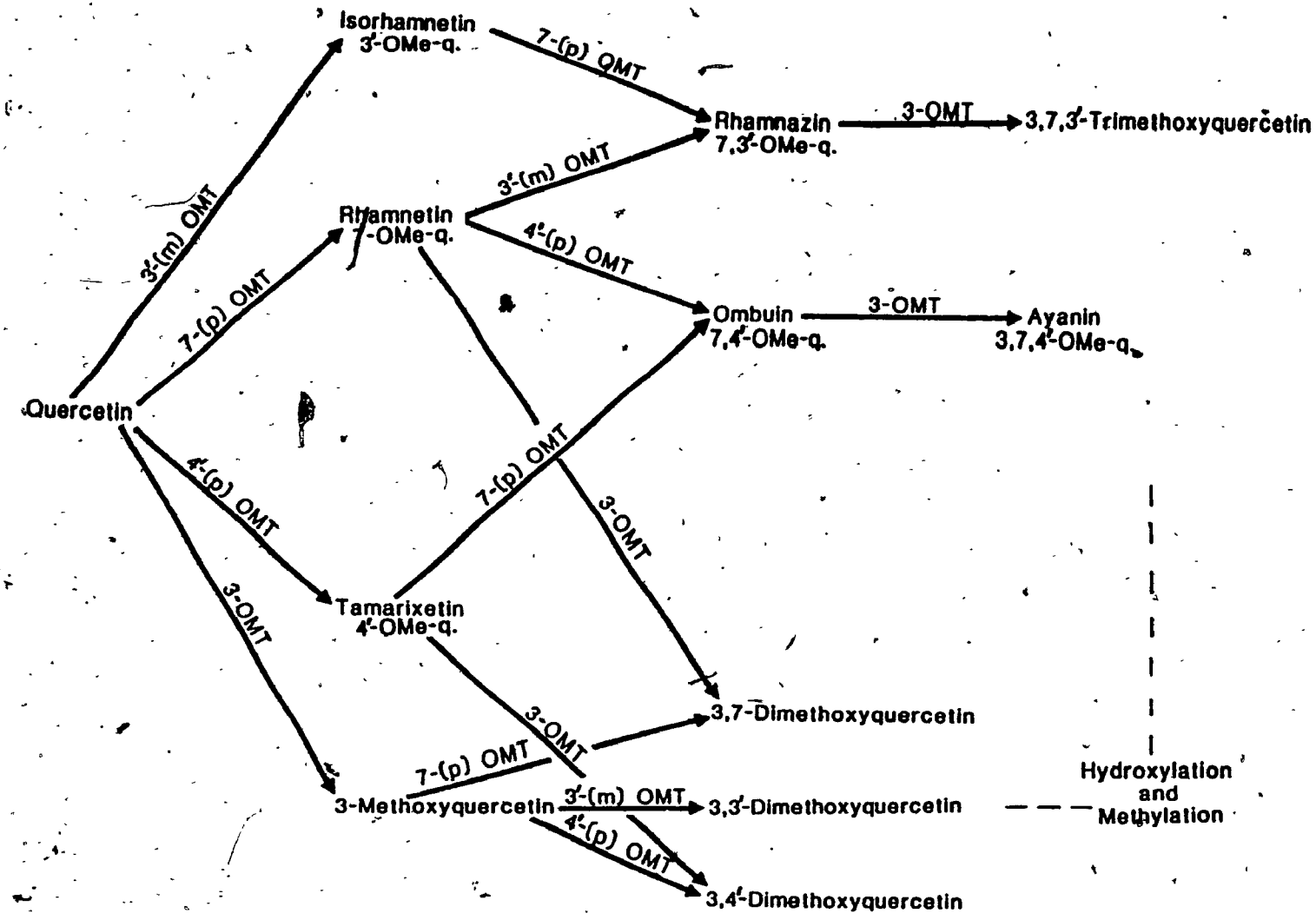


Fig.24 Proposed scheme for the O-methylation of Quercetin

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APPENDIX I

Table VI: *In Vitro* O-Methylation of Flavonoid Compounds with Vicinal Ring-A Hydroxyls[1]

	Rf[2] (x 100)	Total Methylation (%)[3] Root[a]	Citrus Peel[b]	Callus[c]	Tobacco Cells[d]	O-Methyl derivat.	Rf[2] (x 100)	Relative Activity (%)[4] Root	Citrus Peel	Callus	Tobacco Cells	Electron density	OH Position
5,6-Dihydroxyflavone	76p	41	12	n.d.	9	5,6-	87p 70s	35[5]	25[6]	5[7]	5[8]		
						6-	81p 57s	50	30	80	55	-0.1386	6
						5-	78p 49s	5	15	5	5	-0.0865	5
							unidentified	10	30	10	35		
7,8-Dihydroxyflavone	35p	39	20	n.d.	21	7,8-	89p 66s	10[9]	20[10]	20[11]	25[12]		
						8-	78p 53s	65	40	5	5	-0.0369	8
						7-	56p 49s	5	25	5	20	+0.0542	7
							unidentified	20	15	70	50		
5,6,7-Trihydroxyflavone (Baicalein)	41s 0p	165	135	93	54	5,6-/6,7-/5,6,7-	72p 62s 72p	30[13]	55[14]	22[15]	10[16]		
						6-	66p 46s 66p	40	20	22	55	-0.2326	6
						5-/7-	13p 44s 13p	6	5	18	-	-0.1114	7
							unidentified	25	20	38	35	-0.1409	5
5,7,8-Trihydroxyflavone (Norwogonin)	85s	52	71	n.d.	24	7,8-	95s 74p	35[17]	14[18]		-[19]	-0.2089	8
						8-/7-	91s 65p	45	86		100	-0.0819	7
							unidentified	20	-			+0.1843	5

[1] Standard enzyme assay was used as described in the Methods Section
[2] Chromatographed on polyamide (p) or silica gel (s) (Table II)
[3] Based on quercetin as substrate = 100% (a = 15200; b = 18195; c = 13580; d = 13580 cpm/mg/hr)
[4] After separation of individual products by TLC, spots were scraped off the plate and their activities determined by LSC. Total cpms in products 5-19
[5] 7000 cpm; [6] 3500 cpm; [7] 4000 cpm; [8] 8000 cpm; [9] 9000 cpm; [10] 4500 cpm; [11] 4000 cpm; [12] 3500 cpm; [13] 10500 cpm; [14] 3000 cpm; [15] 5000 cpm; [16] 3500 cpm; [17] 6000 cpm; [18] 2500 cpm; [19] 800 cpm
n.d. not determined

Table VII: *In Vitro* O-Methylation of Flavonoids with Vicinal Ring-A Hydroxyls and B-Ring Substitution[1]

	Rf[2] (x 100)		Total Methylation (%)[3] Root[a]	Citrus Peel[b]	Callus[c]	Tobacco Cells[d]	O-Methyl derivat.	Rf[2] (x 100)		Relative Activity (%)[4] Root	Citrus Peel	Callus	Tobacco Cells	Electron density	OH Position
6,7,4'- Trihydroxy-isoflavone	60s	0p	76	47	n.d.	57	6,7,4'-	78s		10^{5}	-		-		
							6,4'-/7,4'-	75s		10	4		8	-0.0985	6-
							6-/7-	70s		50	90^{6}		30	-0.0360	7-
							4'-	63s		15	5		52	-0.0235	4'-
								unidentified		15	5		10		
6,7-Dihydroxy-4'-methoxyiso-flavone (Texasin)	65s	25p	202	66	77	n.d.	Di/Trimethyl	80s		70^{8}		67^{9}		-0.1390	6-
							6,4'/7,4'	76s		10		33		-0.1066	7-
								unjdentified		20		-			
5,6,7,4'- Tetra-hydroxyflavone (Scutellarein)	30s	40c	106	53	60	51	6,4'-	71s	72c	60^{10}	18^{11}	45^{12}	40^{13}	-0.0213	6-
							4'-/6-	65s	56c	30	59	55	45	+0.0984	7-
								unidentified		10	23	-	15	+0.1347	5-
														-0.1395	4'-
5,7,8,4'- Tetra-hydroxyflavone (8-Hydroxyapigenin)	40p		22	40	n.d.	n.d.	8,4'-	64p		37^{14}				-0.2495	8-
							4'-	49p		31				+0.0991	7-
								unidentified		32				+0.2201	5-
														-0.1519	4'-
5,7,8-Trihydroxy-4'-methoxyflavone (8-Hydroxyacacetin)	49p		7	11	n.d.	n.d.	8,4'-/Tri-OCH_3	64p		60^{15}				-0.2089	8-
							7,4'-	59p		2				-0.0819	7-
								unidentified		38				+0.1843	5-
3,5,6,7,3'4'-Hexahydroxyflavone (Quercetagetin)	11s		12	19	n.d.	3	Penta-/Hexa	71s		5^{16}				-0.2969	3-
							3,6,7,3'-	49s		25			30^{17}	+0.0821	6-
							3,6,7,4'-	47s		20			30	+0.0032	7-
							3,6,3'-/3,6,7-	41s		10			-	+0.0846	5-
							Di-/Tri-3,7-	28s		trace					
								22s		trace					
								unidentified		40			60	-0.1086	3'-
														-0.2079	4'-
3,5,7,8,3',4'-Hexahydroxyflavone (Gossypetin)	11s		24	50	n.d.	41	Tetra-/Tri-OCH_3	49s		15^{18}		trace[19]	20^{20}	-0.2956	3-
							Di-/Tri-OCH_3	41s		15		-	-	-0.1059	8-
							Di-OCH_3	38s		10		-	-	-0.1162	7-
								unidentified		60		100	80	-0.2051	5-
														-0.0537	3'-
														+0.1052	4'-

[1] Standard enzyme assay was used as described in the Methods Section
[2] Chromatographed on polyamide (p) or silica gel (s) (Table II)
[3] Based on quercetin as substrate = 100% (a = 15200; b = 18195; c = 27759; d = 13580 cpm/mg/hr)
[4] After separation of individual products by TLC, spots were scraped off the plate and their activities determined by LSC. Total cpms in products 5-19
[5] 12000 cpm; [6] 4500 cpm; [7] 5000 cpm; [8] 7000 cpm; [9] 5000 cpm; [10] 6000 cpm; [11] 7000 cpm; [12] 4000 cpm; [13] 3500 cpm; [14] 4000 cpm; [15] 800 cpm; [16] 2500 cpm; [17] 1000 cpm; [18] 2000 cpm; [19] 1000 cpm; [20] 1500 cpm
n.d. not determined

Table VIII: In Vitro O-Methylation of Flavonoid Compounds Without Vicinal Ring-A Hydroxyls[1]

	Rf[2] (x 100)	Total Methylation (%)[3] Root[a]	Citrus Peel[b]	Callus[c]	Tobacco Cells[d]	O-Methyl derivat.	Rf[2] (x 100)	Relative Activity (%)[4] Root	Citrus Peel	Tobacco Cells	Electron density	OH Position
5,7-Dihydroxyflavone (Chrysin)			SHOWED NO REACTIVITY									
3,5,7-Trihydroxyflavone (Galangin)	75s 53p	23	0	n.d.	160	3,7-	87s	5[5] -	SHOWED	Trace[7]	-0.3273	3
						3-	77s 70p	70 100[6]	NO	100	+0.0684	5
						7-	75s	10 -	REACTIVITY		-0.0127	7
						unidentified	0-29s	15 -				
5,7,4-Trihydroxyflavone (Apigenin)	56s	7	3	2	1	7,4'-	87s	1[8]	1[9]			
						4'-	69s	90	70	0	+0.0349	7
						unidentified	0-29s	9	29		-0.1903	4'
5,7-Dihydroxy-4'-methoxyflavone (Acacetin)	69s	< 1				7,4'-	87s	Trace				
						unidentified	0.29s					
5,7,3',4'-Tetrahydroxyflavone (Luteolin)	31s	165	110	n.d.	n.d.	7,3'-	66s	5[10]	1[11]	n.d.	+0.0293	5
						7,4'-	63s	2	1		+0.0242	7
						3'-	58s	75	87		-0.1217	3'
						4'-	54s	6	8		-0.2176	4'
5,7,4-Trihydroxy-3'-methoxyflavone (Chrysoeriol)	58s	3	n.d.	n.d.	n.d.	7,3'-	65s	Trace	n.d.	n.d.	+0.0069	5
						3'-/3',4'	58s	Trace			+0.0416	7
											-0.1930	4'
3,5,7,4'-Tetrahydroxyflavone (Kaempferol)	55s	6	4	n.d.	n.d.	7,4'-	87s	Trace[12] 0[13]	n.d.	n.d.		
						4'-	70s	40 10			0.3384	3
						7-	65s	10 75			0.3334	5
						3-	60s	20 5			0.0028	7
						unidentified	0-29s	10 5			+0.1537	4

[1] Standard enzyme assay was used as described in the Methods Section
[2] Chromatographed on polyamide (p) or silica gel (s) (Table II)
[3] Based on quercetin as substrate = 100% (a = 15208; b = 18195; c = 27759; d = 13580 cpm/mg/hr)
[4] After separation of individual products by TLC, spots were scraped off the plate and their activities determined by LSC. Total cpms in products 5-19
[5] 5000 cpm; [6] 5000 cpm; [7] 8000 cpm; [8] 1500 cpm; [9] 2000 cpm; [10] 10000 cpm; [11] 3200 cpm; [12] 15000 cpm; [13] 3000 cpm
n.d. not determined

Table IX: In Vitro O-Methylation of Flavonoid Compounds of the Quercetin Series[1]

	Rf[2] (x 100)	Total Methylation (%)[3] Root[a]	Citrus Peel[b]	Citrus Callus[c]	Tobacco Cells[d]	O-Methyl derivat.	Rf[2] (x 100)	Relative Activity (%) Root	Citrus Peel	Citrus Callus	Tobacco Cells	Electron density	OH Position
3,5,7,3',4'-Pentahydroxyflavone (Quercetin)	28s 45c 18p 0p	100	100	100	100	7,3'-/7,4'-	65s 62c 48/55p	31[5]	12[6]	25[7]	5[8]	-0.3090	3
						3,7-/3,3',4'-	59s	2	-	-	1	+0.0474	5
						3'-/3,3'-/3,4'-	56s 8/17p	16	54	5	0	-0.0162	7
						3-/4'-	53s 55c 11/11p	9	17	5	0	-0.1100	3'
						3-	39s	3	3	3	2	+0.2089	4'
						5-	32s	10	-	-	6		
						Unidentified	0-25s	29	14	63	86		
5,7,3',4'-Tetrahydroxy-3-methoxyflavone (3-Methylquercetin)	39s 8p	115	n.d.	n.d.	14	3,7-	59s 46p	65[9]			66[10]	-0.0287	5
						3,3'-/3,4'-	56s	30			8	-0.0002	7
						Unidentified	0-10s	5			26	+0.0747	3'
												0.1578	4'
3,5,3',4'-Tetrahydroxy-7-methoxyflavone (Rhamnetin)	51s 53c 11p 22p	64	73	33	64	Tri-Tetra-OCH_3	70-80s	trace[11]	-[12]	-[13]	0[14]	-0.3093	3
						3,7,4'-/3,7,3'-	67s	17	-	-	2	-0.0477	5
						7,4'-/7,3'-	65s	41	30	40	13	+0.1092	3'
						3,7-	59s 46p	16	-	-	6	-0.2079	4'
						3'-	57s 17p	3	15	-	6		
						7-	52s 22p	5	23	-	6		
						Unidentified	0-24s	18	17	60	67		
3,5,7,4'-Tetrahydroxy-3'-methoxyflavone (Isorhamnetin)	57s 49c 8p 17p	47	25	20	34	Tri-/Tetra-OCH_3	68-80s	trace[15]	-[16]	-[17]	-[18]	-0.3240	3
						7,3'-	65s	17	7	2	13	+0.0328	5
						3,3',4'-	59s	5	-	-	6	-0.0037	7
						3'-/3,3'-	57s	8	45	-	11	-0.1768	4'
						7-	52s	10	18	-	-		
						Unidentified	0-24s	60	30	98	70		
3,5,7,3'-Tetrahydroxy-4'-methoxyflavone (Tamarixetin)	50s 55c 11p	79	54	n.d.	39	7,4'-	65s	75[19]		n.d.	58[20]	+0.3262	3
						3,4'-	55s	4			16	-0.0326	5
						Unidentifed	0-24s	21			26	+0.0033	7
												+0.0934	3'
3,5,3'-Trihydroxy-4'-dimethoxyflavone (Ombiun)	65s 62c 48p 55p	32	17	n.d.	5	3,7,3',4'-	73s 95p	1[21]		n.d.	-[22]		
						3,7,4'-	68s 79p	49			69	+0.3258	3
						7,4'-/7,3',4'-	64s	37			9	-0.0327	5
						Unidentified	0-25s	13			31	-0.0932	3'
3,5,7-Trihydroxy-3',4'-dimethoxyflavone (3',4'-dimethyl-quercetin)	57s 81c 33p	12	n.d.	n.d.	3	7.3'.4'-	68s	43[23]		n.d.	30[24]	-0.3080	3
						Internal substr.	63s	2			-	+0.0358	5
						3,3',4'-	60s	7			37	+0.0053	7
						3',4'-	58s	33			15		
						Unidentified	0-25s	17			18		

[1] Standard enzyme assay was used as described in the Methods Section
[2] Chromatographed on polyamide (p) or silica gel (s) (Table II)
[3] Based on quercetin as substrate = 100% (a = 15200; b = 18195; c = 27759; d = 13480 cpm/mg/hr)
[4] After separation of individual products by TLC, spots were scraped off the plate and their activities determined by LSC. Total cpms in products 5-19
[5] 16500 cpm; [6] 5000 cpm; [7] 5000 cpm; [8] 12000 cpm; [9] 10000 cpm; [10] 4000 cpm; [11] 21000 cpm; [12] 7000 cpm; [13] 2500 cpm; [14] 6000 cpm; [15] 12500 cpm; [16] 4000 cpm; [17] 5000 cpm; [18] 3200 cpm; [19] 23250 cpm; [20] 5700 cpm; [21] 20250 cpm; [22] 1000 cpm; [23] 10750 cpm; [24] 3200 cpm
n.d. not determined

APPENDIX II

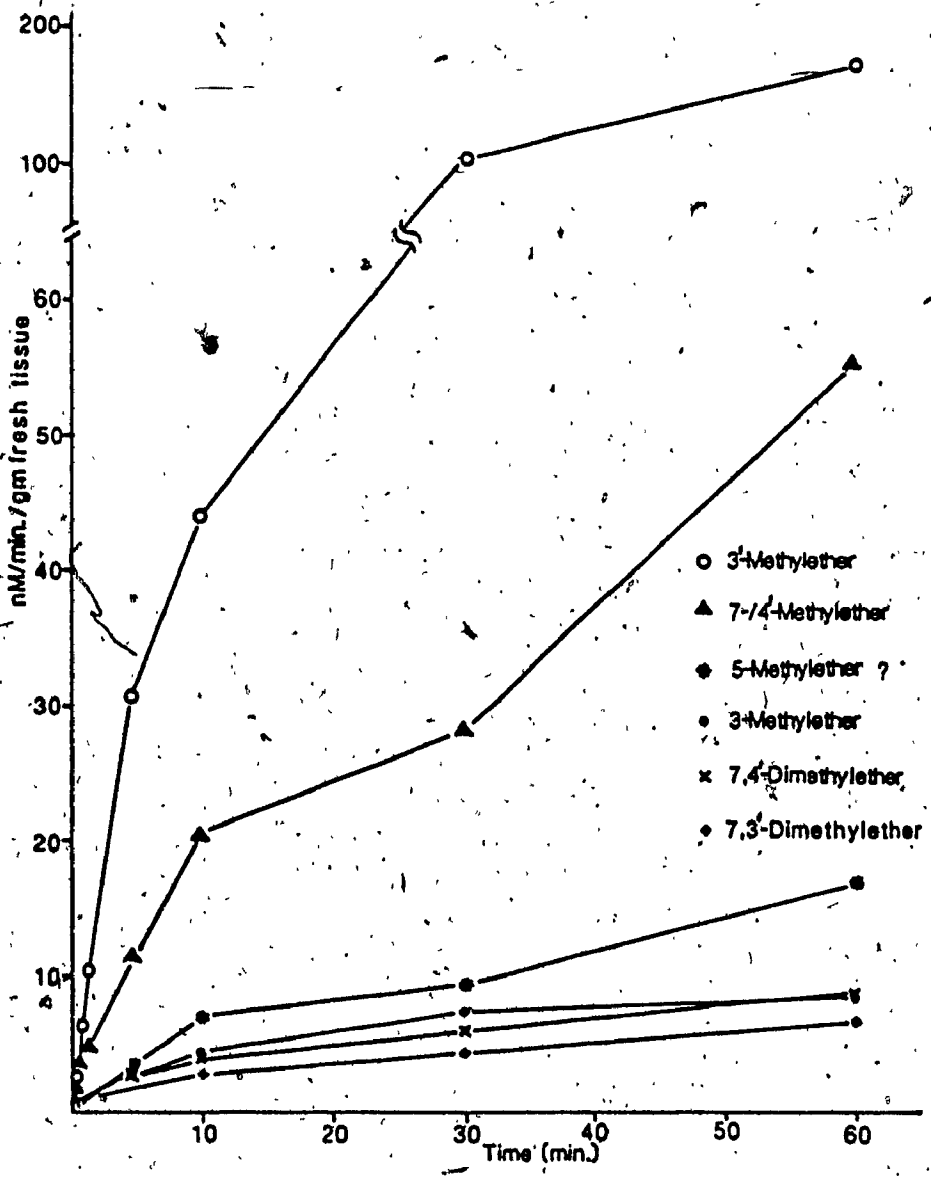

Fig.20 Time-course of product formation with quercetin as substrate

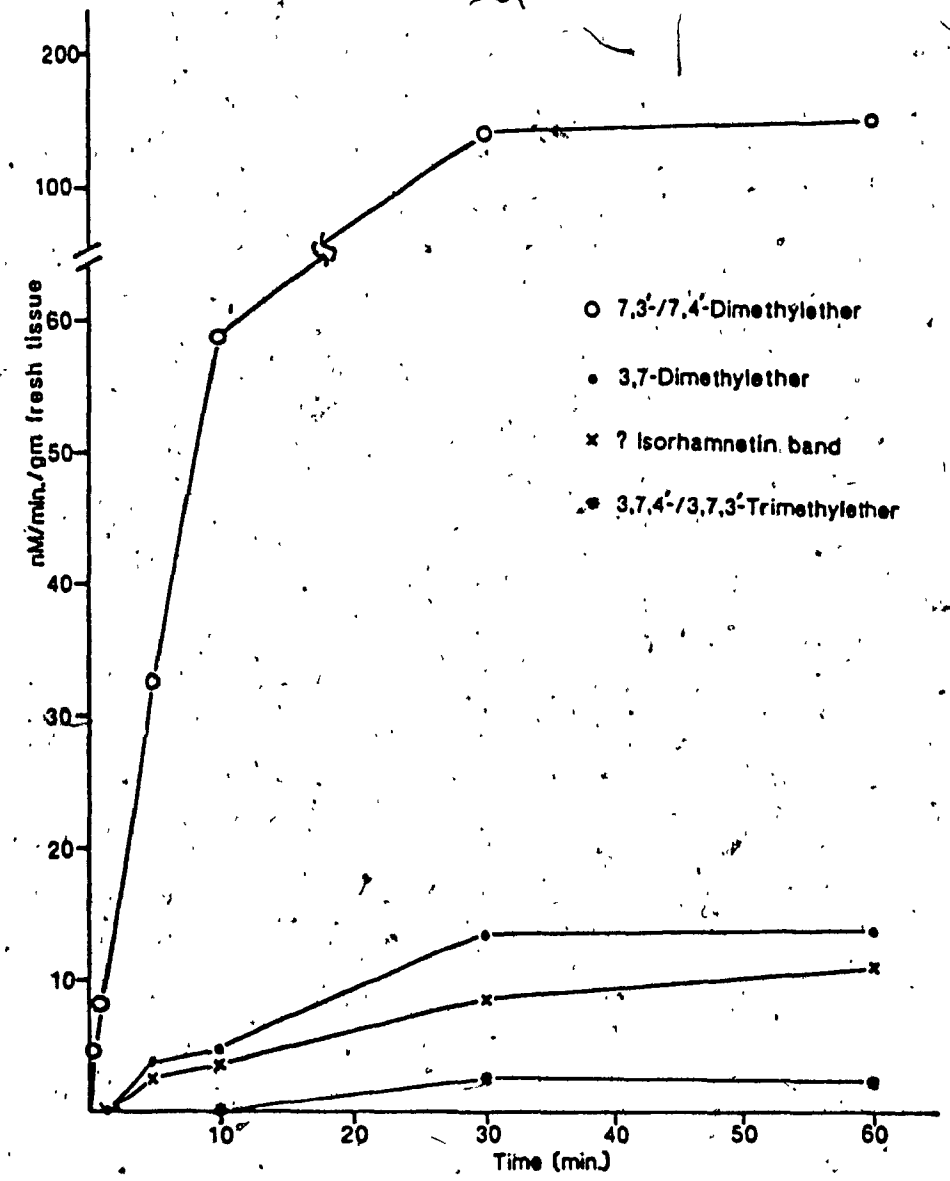

Fig.21 Time-course of product formation with Rhamnetin as substrate

APPENDIX III

Some aspects of the kinetics of the methylation reaction

Although a stable purified enzyme could not be obtained with citrus, an attempt was made to establish Km values with the semi-purified enzyme, with a view to compare these values with those in the literature. Due to the nature of the enzyme(s) and the occurrence of multiple product formation by step-wise methylation of the substrate, the respective Km's were obtained from saturation curves (Fig. 27-29) and their respective Lineweaver-Burk plots (Fig. 27a-29a). The non-linearity of the Lineweaver-Burk transformation (except for luteolin) demonstrates that the enzyme reaction is complex and that the Km's therefore represent only approximate values.

Determination of the Michaelis constant (Km) for 3,5,6,3',4'-Pentahydroxyflavone (Quercetin), 3,5,3',4'-Tetrahydroxy-7-methoxxyflavone (Rhamnetin), 6,7,4'-Trihydroxyisoflavone and 5,7,3',4'-Tetrahydroxyflavone (Luteolin)

Substrate	Approximate Km
Quercetin	2.3 - 7.4 x 10^{-5} M (Fig. 27)
Rhamnetin	2.3 - 7.5 x 10^{-5} M (Fig. 28)
6,7,4'-Tri-OHisoflav.	6.3 - 24.5 x 10^{-5} M
Luteolin	0.8 - 1.3 x 10^{-5} M (Fig. 29)

The approximate Km values for the citrus enzyme fall within the range of Km's reported in the literature.

Substrate	Source	Km	Reference
Quercetin	Tulip	17 x 10^{-5}M	Sütfeld and Wiermann (1978)
	Chrysosplenium	0.1-8x10^{-5}M	DeLuca and Ibrahim (1982) Ibrahim et al. (1982)
	Lotus	2.2 x 10^{-5}M	Jay et al. (1983) Ibrahim et al. (1982)
7,4'-Dihydroxy-isoflavone	Cicer	8 x 10^{-5}M	Wengenmeyer et al. (1974)
5,7,3',4'-Tetra-Hydroxy-flavonone	Parsley	1.2 x 10^{-5}M	Ebel et al. (1972)
Luteolin	Parsley	4.6 x 10^{-5}M	Ebel et al. (1972)

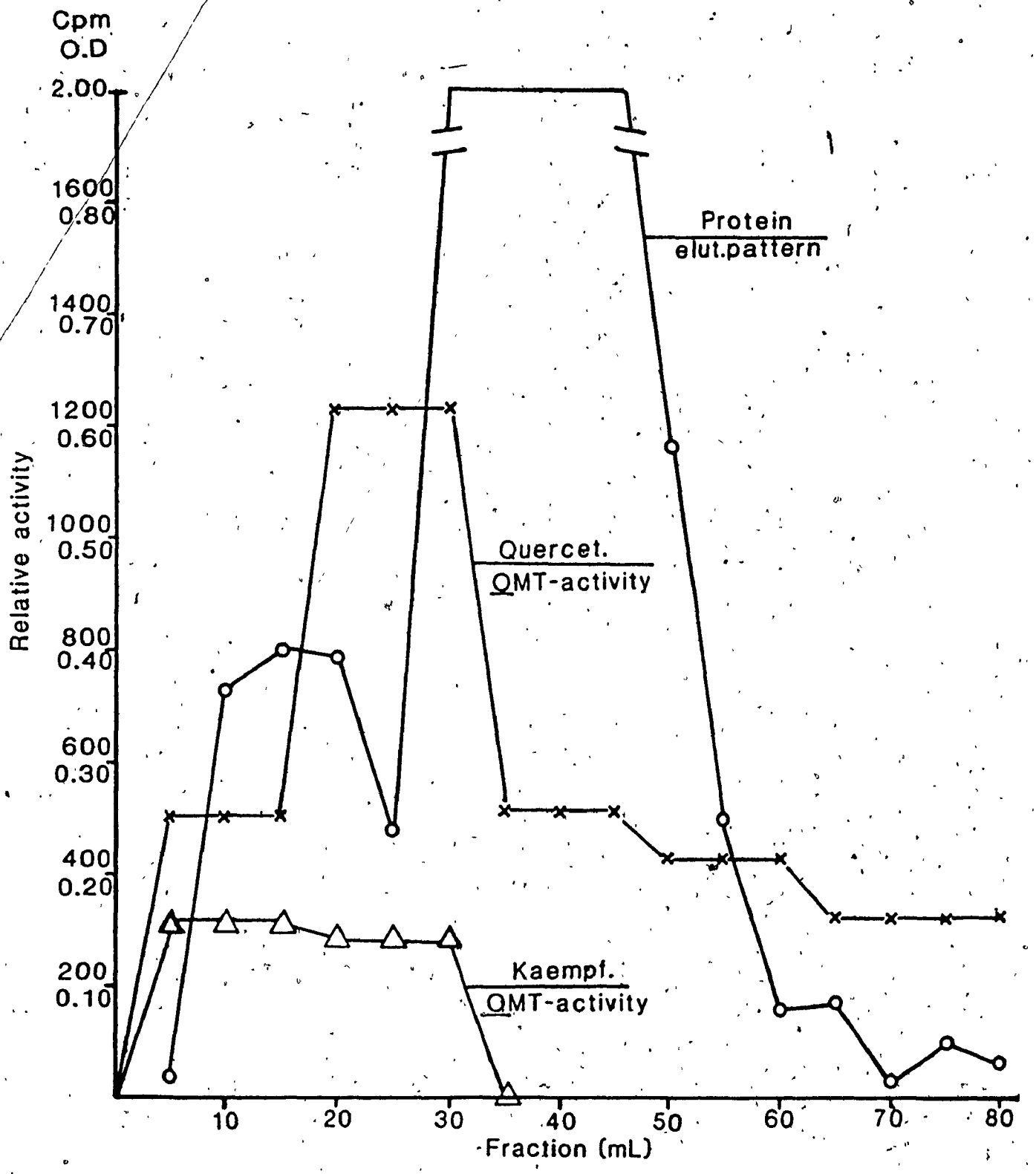

Fig.25 Elution profile and QMT activity of citrus root extract chromatographed on Sephadex G-100

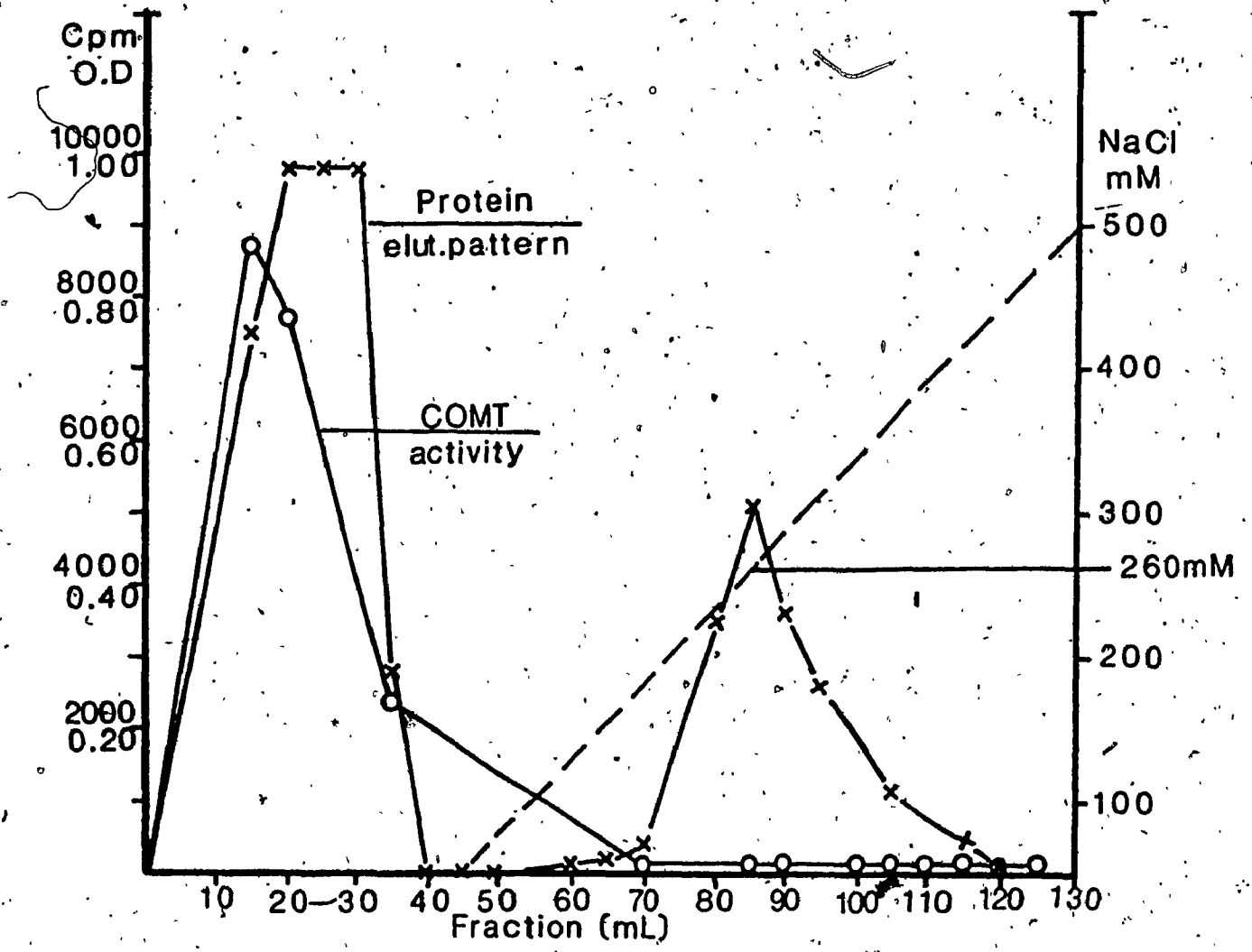

Fig.26 Elution profile and OMT activity of citrus root extract chromatographed on quercetin ligand

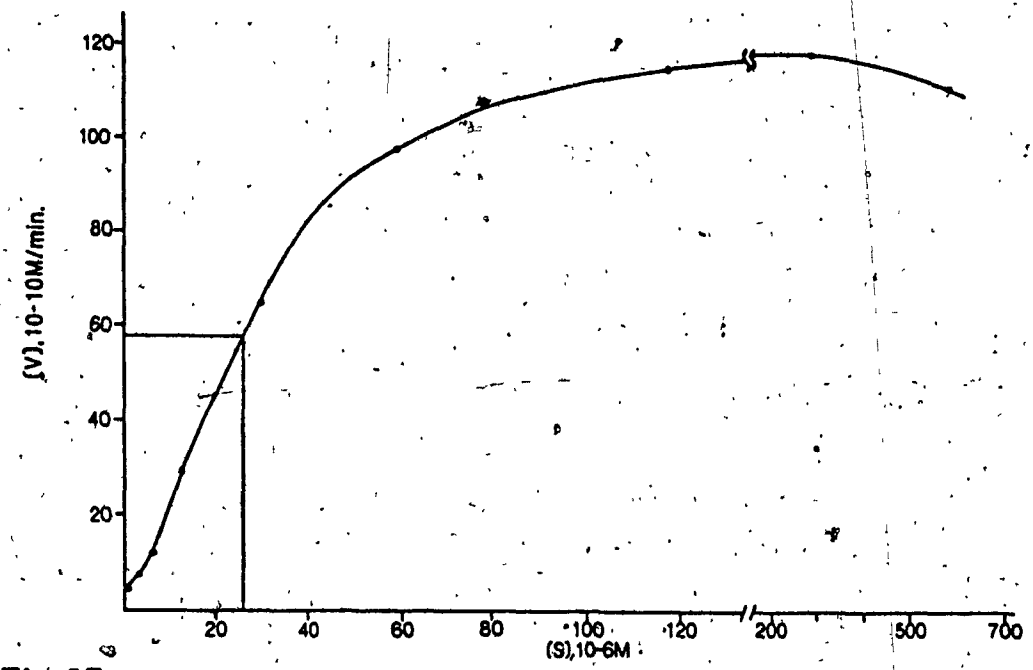

Fig.27 Determination of the apparent Km for Quercetin

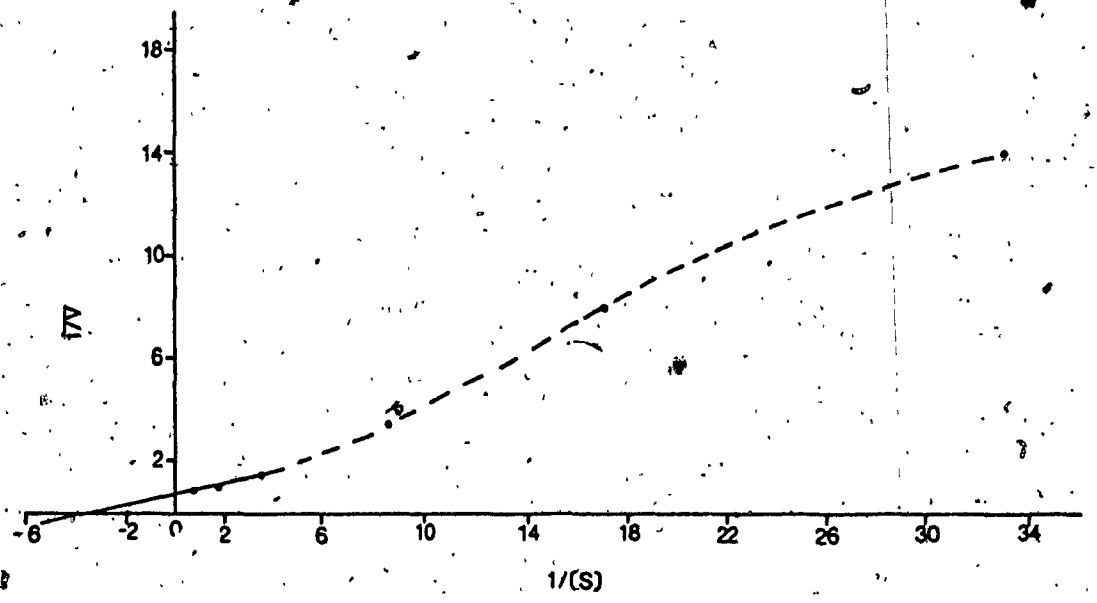

Fig.27a Lineweaver-Burk plot of the Quercetin curve

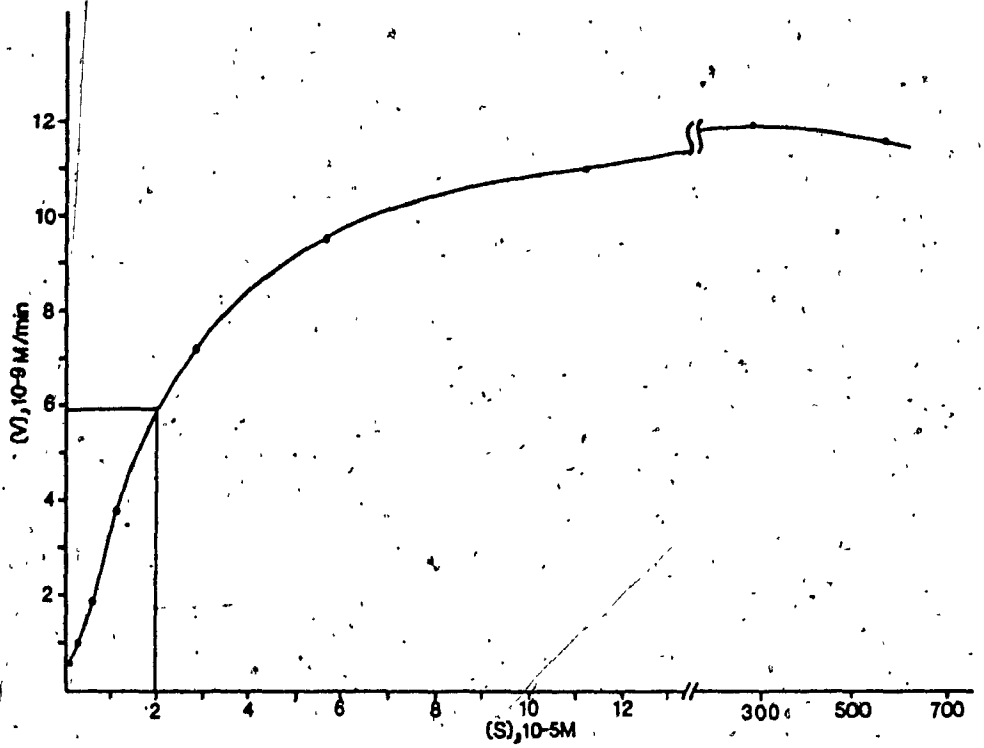

Fig.28 Determination of the apparent Km for Rhamnetin

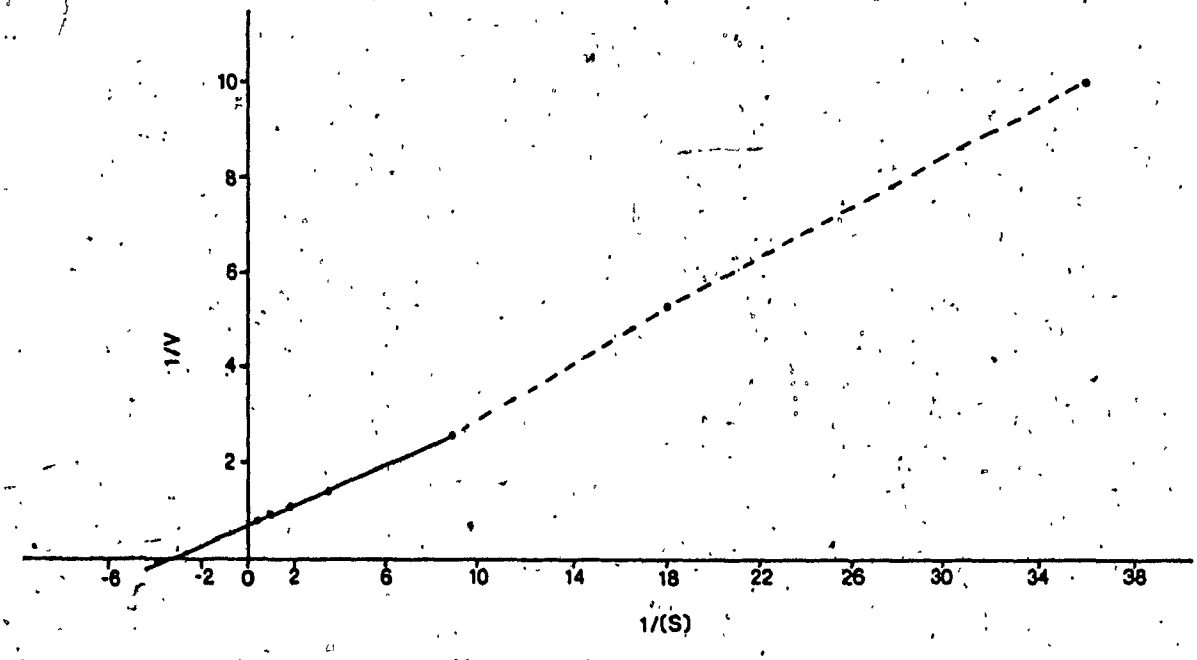

Fig.28a Lineweaver-Burk plot of the Rhamnetin curve

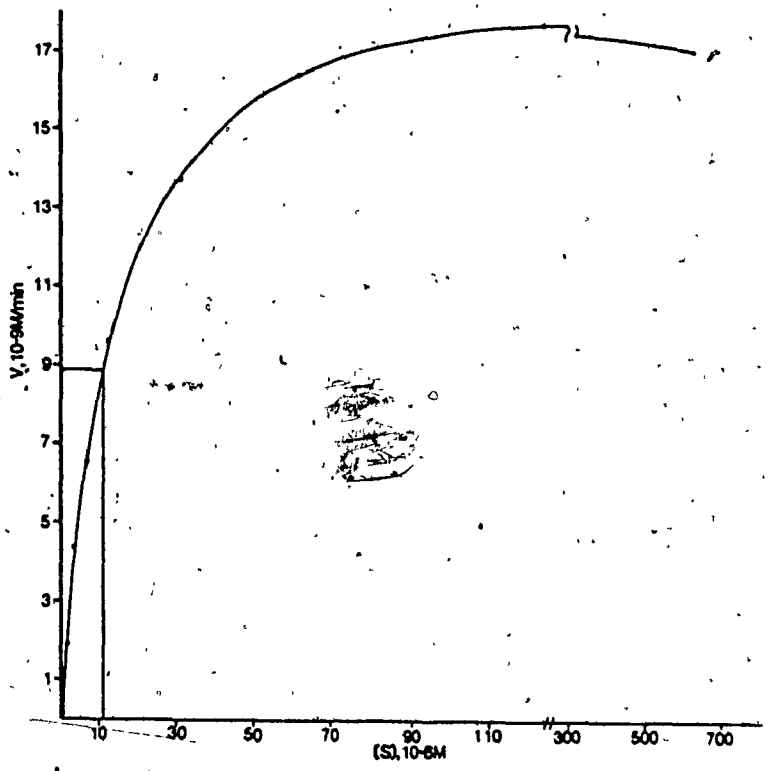

Fig.29 Determination of the apparent Km for Luteolin

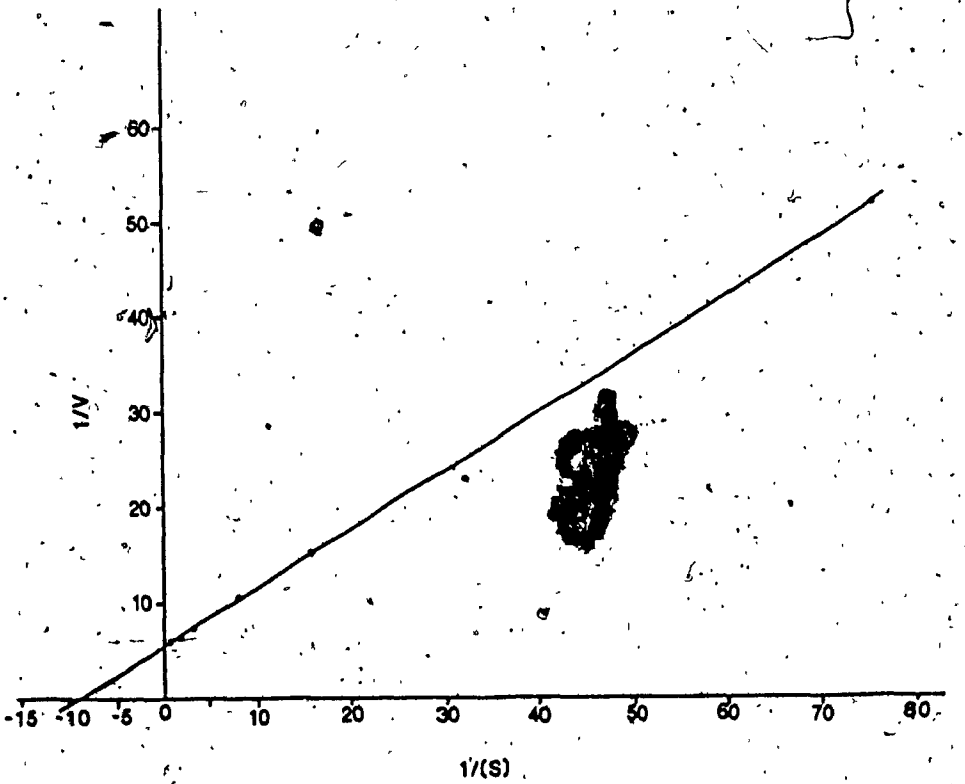

Fig.29a Lineweaver-Bürk plot of the Luteolin curve